ACTIVITY AND KINETIC INVESTIGATIONS ON GLUCOSE-6-PHOSPHATE DEHYDROGENASE AND ALCOHOL DEHYDROGENASE IN MICROCAPTIVE ENVIRONMENT IN NON-AQUEOUS SOLVENTS

A Thesis Submitted
in Partial Fulfilment of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

By

TAPAS K. DE

to the

DEPARTMENT OF CHEMISTRY

INDIAN INSTITUTE OF TECHNOLOGY, KANPUR

APRIL, 1989

Dedicated To My

Family

STATEMENT

I hereby declare that the matter embodied in this thesis entitled **"ACTIVITY AND KINETIC INVESTIGATIONS ON GLUCOSE-6-PHOSPHATE DEHYDROGENASE AND ALCOHOL DEHYDROGENASE IN MICROCAPTIVE ENVIRONMENT IN NON-AQUEOUS SOLVENTS"** is the result of investigations carried out by me in the Department of Chemistry, Indian Institute of Technology, Kanpur, India, under the supervision of Professor S.S. Katiyar.

In keeping with scientific tradition, due acknowledgement has been made wherever the work described is based on the finding of other investigators.

Tapas K. De

CERTIFICATE I

Certified that the work presented in this thesis entitled "**ACTIVITY AND KINETIC INVESTIGATIONS ON GLUCOSE-6-PHOSPHATE DEHYDROGENASE AND ALCOHOL DEHYDROGENASE IN MICROCAPTIVE ENVIRONMENT IN NON-AQUEOUS SOLVENTS**" by Mr. Tapas K. De has been carried out under my supervision and the same has not been submitted elsewhere for a degree.

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CERTIFICATE II

This is to certify that Mr. Tapas K. De has satisfactorily completed all the courses required for the Ph.D. degree programme. These courses include:

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Chm 521N	Chemical Binding
Chm 525N	Principles of Physical Chemistry
Chm 545N	Principles of Inorganic Chemistry
Chm 622N	Chemical Kinetics
Chm 524N	Modern Methods in Physical Chemistry
Chm 800N	General Seminar
Chm 801N	Special Seminar
Chm 900N	Research

Mr. Tapas K. De was admitted to the candidacy of Ph.D. degree programme in January 1985 after he successfully completed the written and oral qualifying examinations.

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ACKNOWLEDGEMENTS

With great pleasure, I express my profound sense of gratitude to my thesis supervisor, Professor S.S. Katiyar, for his able and dynamic guidance, elaborate advice, inspiration and constructive criticism during the entire course of this work. It is a pleasure to acknowledge my personal and intellectual debt to him.

I extend a very special thanks to all the faculty members of this department in general and Professor U.C. Agarwala, Professor P.C. Nigam and Professor S.K. Dogra in particular for their kind interest and encouragement.

My personal and deep appreciation goes to my colleagues Dr. Anil Kumar, Mr. Ajay Kumar, Ms. Amita Srivastava, Ms. Manisha Borwankar, Dr. Manoj Kumar Srivastava and Mr. Neelay Dedhia for their selfless co-operation and warm friendliness during my stay. I am also grateful to my other colleagues of this department for their help and co-operation in various matters.

I express my heartiest thanks to all my friends especially to Drs. Hemant Kumar Sinha, Maheswar Roy and Mr. V.K. Shankaranarayanan for their encouraging and cheerful company throughout my stay in IIT Kanpur.

I am thankful to the IIT Kanpur for providing me financial assistance and other facilities during the research work.

I feel attachment and extend thank to Sri Har Narayan Singh for his hospitality, co-operation and help in his own way provided to me during my stay in the laboratory.

I am thankful to Mr. Anil Kumar Jauhari for typing thesis and other papers, to Messers J.S. Rawat, G.P. Shukla and L.P. Tripathi for procuring the chemicals, Mr. R.K. Bajpai for drawing the figures, Mr. B.N. Shukla for technical help and several other staff members of this institute for their co-operation during my research work.

I am deeply indebted and express my deepest sense of gratitude to my parents and other family members for their sacrifice and encouragement throughout my career.

Tapas K. De

SYNOPSIS

Enzymes are of paramount importance for cell metabolism. Their highly specific and extremely efficient catalytic power leads to extensive prospects for the employment of enzymes in technological processes. Studies of enzymes *in vitro* are usually conducted in buffered aqueous medium. However in the living cell, enzymes function on or near the water/membrane interface. Some of them are located on the surface of biological membranes or inside them. In principle, there is no doubt that physical properties (and especially the dielectric permeability) of water in proximity to the interface differ significantly from those of bulk water. That is why some researchers are inclined to believe that the traditional enzymology (studying the properties of enzymes in water solutions) may not give real characteristics of some enzymes. In order to look for an alternative medium for study of enzyme action *in vitro* and to extend the utility of enzymes in technological processes, specially in organic syntheses, which are thermodynamically favoured in organic solvent, it will be desirable to use them in organic solvent. For this purpose some means must be found out to protect the enzymes from the harmful effects of organic solvent. A reasonable way out of this situation is the solubilization of enzymes in organic solvents with the aid of surfactants and small amount of water. The surfactant molecular aggregates in organic solvent yield reverse micelles. On solubilization, the enzyme is entrapped into the inner cavity of reverse micelle containing

water. This water pool inside the core of reverse micelle behaves as a microreactor where enzymes are protected from the harmful effects of organic solvent. Thus enzymes are made to work in the microcaptive environment in non-aqueous solvents. It is found in recent years that reverse micelles of synthetic surfactants provide an unique microcaptive environment in non-aqueous solvents for the action of enzymes, nucleic acids and other biomolecules.

First chapter deals with the introduction of the subject as well as a brief and critical review of the literature of normal micelle, reverse micelle and micellar enzymology. Description of technological and biotechnological applications has been given special emphasis as there are immense prospects of exploiting micellar enzymology in these areas. Objective of the present work has been analysed briefly in this chapter.

The next three chapters present the experimental study of enzymes glucose-6-phosphate dehydrogenase and alcohol dehydrogenase in reverse micellar solution in organic solvent. So far few enzymes which were simple and having low molecular weight and single subunit, were investigated in reverse micelles. Therefore in the present work in reverse micellar microcaptive environment in non-aqueous solvents a class of oxidoreductase enzymes has been chosen. Additional reason to select the enzymes of this class was due to their diverse characteristics such as having more than one subunit, possession of complexity of involvment in coupled reactions and having very specific and

important use in many biological and technological processes. Investigations on dehydrogenases in such kind of microcaptive environment, are likely to provide a realistic model of enzyme behavior in cellular environment as the hydrophobic and polar environment provided by the reverse micelles is somewhat similar to the cellular environment.

In the second chapter are reported investigations on glucose-6-phosphate dehydrogenase from yeast. Glucose-6-phosphate dehydrogenase is the key enzyme in the pentose phosphate cycle. The enzyme catalyses the oxidation of glucose-6-phosphate to 6-phosphoglucono-δ-lactone.

$$\text{Glucose-6-phosphate} + NADP^{+} \xrightarrow{\text{G-6-PDH}} \text{6-phosphoglucono-}\delta\text{-lactone} + NADPH + H^{+}$$

This enzyme is relatively big in size. It has a molecular weight of 212,000 dalton in presence of NADP with four sub-units. It is used for enzymatic determination of NADP, glucose-6-phosphate, glucose-1-phosphate. It is important for the determination of activity of phosphoglucose isomerase, phosphoglucomutase and hexokinase.

For the study of yeast glucose-6-phosphate dehydrogenase in reverse micelles, the system bis(2-ethylhexyl) sodium sulfosuccinate, Aerosol OT(AOT) and polyoxyethylene(5)octylphenol (Triton X-45) mixture (1:1 molar ratio) in n-heptane non-aqueous medium has been used. The reverse micellar solution of mixed

surfactant was chosen because this enzyme was found to be inactive in cationic (cetyltrimethylammonium bromide (CTAB), anionic (AOT) or non-ionic (Triton X-45) surfactants separately. The choice of these surfactant systems is also based on their special characteristics to provide a bigger water pool size. The size of the water pool can be varied either by changing water content or surfactant concentration. The molar ratio of water to surfactant concentration is called degree of hydration i.e. W_o(W_o = $[H_2O]/[surfactant]$). The W_o is an important parameter which determines most of the structural and physical properties of the reverse micelle. Solubilization of glucose-6-phosphate dehydrogenase in this reverse micellar system depends on surfactant concentration, the degree of hydration (W_o), type, concentration and pH of buffers, concentration of enzymes, temperature etc. Injection method was followed to solubilize this enzyme in this microheterogeneous system.

It was found that the enzyme activity depended on parameters like W_o, pH and total surfactant concentration and ratio of surfactant concentrations etc. The maximum enzyme activity was observed at the optimum conditions of W_o, pH and surfactant concentration. One of the most striking feature of glucose-6-phosphate dehydrogenase activity in this reverse micellar system, is the appearance of superactivity at W_o 55.56, pH 9.7, 0.05M AOT and 0.05M Triton X-45. At this condition the enzyme activity is 233% than that in aqueous solution at optimum conditions.

Next we carried out spectral study to establish the identical nature of the enzyme reaction in aqueous buffered medium and micellar solution. For this purpose the absorption spectra of the aqueous and reverse micellar solution, before and after the completion of enzyme reaction, were recorded. The spectral data obtained at different values of W_o established the formation of same product in both the media.

Investigation on storage and operational stability of glucose-6-phosphate dehydrogenase in this microcaptive environment is of great interest because it can improve the storage condition of this enzyme. The solubilized enzyme was stored in the reverse micelle in the absence and presence of coenzyme and substrate at 30°C. The residual activity was checked at different intervals of time. It was found that glucose-6-phosphate dehydrogenase when incubated with $NADP^+$ shows better stability than the enzyme incubated with substrate or in the absence of substrate and coenzyme. Stability was also dependent on W_o. Glucose-6-phosphate dehydrogenase shows better stability under conditions where it shows maximum activity.

In the last part of chapter II we have investigated effect of substrate concentration, coenzyme concentration and enzyme concentration on the reaction of glucose-6-phosphate dehydrogenase in the microheterogeneous medium. Kinetic parameters and kinetic characteristics for this enzyme in this medium have been determined. In this medium substrate concentration effect showed that the enzyme obeyes Michaelis-

Menten kinetics up to a specific concentration range of substrate and at high substrate concentration it exhibits substrate inhibition. Effect of co-enzyme concentration shows that the enzyme follows characteristics saturation kinetics. Further the enzyme in micellar medium follows initial velocity patterns (Lineweaver-Burk plots) similar to those obtained in aqueous medium. Michaelis constant (K_m) and other kinetic and binding parameters were calculated from the primary and secondary Lineweaver-Burk plots. K_m in micellar medium can be expressed in two ways; (i) $(K_m)_{overall}$ abbreviated as $(K_m)_{ov}$ (ii) $(K_m)_{water\ pool}$ abbreviated as $(K_m)_{wp}$. When it is considered that the concentration of substrates and coenzymes are in overall volume of micellar solution then it gives $(K_m)_{ov}$ and if concentration of substrates and coenzymes is considered to be restricted in the water pool only then it gives the value of $(K_m)_{wp}$. For glucose-6-phosphate dehydrogenase $(K_m)_{ov}$ for substrate and coenzyme are 6.896×10^{-5}M and 3.22×10^{-5}M respectively.

Chapter III presents an exhaustive investigation on activity and kinetic study of alcohol dehydrogenase from yeast (YADH) in reverse micellar medium. It is a complex enzyme having M.W. ~ 151,000 dalton with 4 sub-units. Alcohol dehydrogenase catalyzes the oxidation of alcohols and the reduction of aldehydes and ketones in the presence of NAD and NADH respectively.

$$RCH_2OH + NAD^+ \xrightleftharpoons{YADH} RCHO + NADH + H^+$$

Yeast alcohol dehydrogenase has a more narrow specificity than does the horse liver enzyme. YADH accepts ethanol as substrate, is less active on other straight chain primary alcohols, and acts to a very limited extent on certain secondary and branched chain alcohols. NADP does not serve as co-enzyme. The common reaction in yeast cells is the reduction of acetaldehyde to ethanol by YADH. YADH is used analytically for the determination of ethanol and as a coupling enzyme for other reactions which yield ethanol, such as the hydrolysis of benzonyl-1-arginine ethyl ester (BAEE).

For the study of YADH in reverse micelles, the system anionic surfactant bis(2-ethylhexyl) sodium sulfosuccinate (AOT) in isooctane was found to be suitable. The enzyme remains active, rather shows superactivity in this microheterogeneous medium under specific conditions of W_o, pH and surfactant concentration. The selection of this surfactant was also based on its special characteristics to provide a bigger water pool to accommodate the big enzyme YADH. In this case too the solubilization is governed by the parameters W_o, AOT concentration, type and concentration of buffer. The effect of W_o, pH and surfactant concentration on the activity of yeast alcohol dehydrogenase in this microheterogeneous medium was carried out systematically to find out optimum conditions for the maximum activity of this enzyme in this non-aqueous solvent. It was found that the enzyme shows maximum activity at the condition

where W_o = 20.56, pH = 10.7 and [AOT] = 0.15M. The activity of the enzyme is 172% than that of its activity in aqueous buffer. This demonstrates that this enzyme is superactive in this medium. Spectral data of the enzyme reaction cocktail before and after the completion of the reaction established the formation of same product in both the media. Experiments on storage and operational stability of the enzyme in reverse micelle showed that YADH like glucose-6-phosphate dehydrogenase was more stable when incubated with co-enzyme NAD^+.

Last part of Chapter III describes the effect of concentration of co-enzyme, substrate and enzyme on YADH activity in the microheterogeneous medium of AOT/isooctane. These data showed that the enzyme obeyes Michaelis-Menten kinetics in this reverse micellar medium. The characteristic constants (K_m) and other kinetics and binding parameters) of the enzyme in this microheterogeneous medium at the condition of maximum enzyme activity were calculated from the primary and secondary Lineweaver-Burk plots. $(K_m)_{ov}$ for both the substrate and co-enzyme was found to be 11.764×10^{-2}M and 1.0×10^{-3}M respectively. As $(K_m)_{ov}$ is considered to be good measure of dissociation constant of the enzyme-substrate (E-S) complex in micellar medium, the stability of the E-S complex in this reverse micellar medium remains low.

Chapter IV deals with the experiments on yeast alcohol dehydrogenase in reverse micellar solution of cationic surfactant cetyltrimethylammonium bromide (CTAB). YADH when solubilized in

cationic reverse micelles of CTAB in isooctane-$CHCl_3$ (1:1) maintains its activity. The solubilization of this enzyme in CTAB/isooctane-$CHCl_3$ (1:1, v/v) was highly regulated by the parameters like W_o, pH, enzyme concentration, surfactant concentration etc. To optimize the conditions for maximum activity of the enzyme in this reverse micellar medium effect of (W_o), pH, surfactant concentration was studied systematically. It was observed that the maximum activity was only 47% than that of aqueous buffer at W_o = 15.00, pH = 10.00 and [CTAB] = 0.1M.

Spectral study on the nature of enzyme reaction in aqueous and reverse micelle solution showed no change in product characteristics. Time dependent stability of this enzyme in this microheterogeneous medium was determined by incubating the enzyme in different combination of co-enzyme and substrate. The enzyme was found to be very unstable when put with C_2H_5OH. It seems that though C_2H_5OH is substrate it denatures the enzyme. The enzyme shows better stability with co-enzyme NAD^+.

Lastly we have investigated the effect of substrate concentration, co-enzyme concentration and enzyme concentration on the activity of YADH in this organic solvent. It was found that though co-enzyme followed Michaelis-Menten kinetics, substrate did not. The activity of the enzyme was obtained after certain concentration of substrate and it did not change with increase of substrate concentration. The values of $(K^{NAD})_{ov}$ was found to be unusually higher compared to aqueous medium.

These data on the study of glucose-6-phosphate dehydrogenase and yeast alcohol dehydrogenase show that these enzymes are able to retain their catalytic activity while solubilized in reverse micelles and thus maintain their conformational integrity and subunit-subunit interaction inside the micellar core. As some features of reverse micelles are similar to those of biomembranes, display of superactivity by glucose-6-phosphate dehydrogenase and yeast alcohol dehydrogenase shows that enzymes *in vivo* may possess higher activity than actually found by in *in vitro* studies in aqueous solution.

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CHAPTER I

GENERAL INTRODUCTION

Enzymes are highly specialized protein molecules made in cells from simple amino acids. They are biological catalysts possessing extraordinary efficiency and substrate specificity. They bring out their remarkable catalytic power in dilute aqueous solution at biological pH and moderate temperature, in sharp contrast to rather extreme conditions often required for non-enzymatic reactions in the laboratory. Enzymes are intimately involved in the transformation of different forms of energy. They are catalysts and consequently can not alter the equilibrium of a chemical reaction. An enzyme accelerates the forward and reverse reaction by precisely the same order. They accelerate reactions by decreasing the activation energies of reactions catalysed by them by factors of the order of a million. In cells they have paramount role in carring out the functions of cell wall, cell membrane, nucleus, mitochondria, chloroplast, ribosome, endoplasmic reticulum, golgi body etc. In short, enzymes are essential to life. A thorough investigation and understanding of their function and control of reactions are of supreme importance towards any understanding of life.[1-6]

I.1 Enzyme Study in Buffer

In vitro study of the properties and behavior of an enzyme as a chemical catalyst has been carried out in the laboratory in aquous buffer medium. The most valuable experiments aimed at elucidating the structure of catalytic centres and the physico-chemical mechanism of biocatalysts were successful only with the enzymes isolated from the living cells in pure form.[1,4, 7, 8] In the test tube environment around enzyme is very different from its natural environment in the cell. It should not be overlooked whether the enzyme properties observed *in vitro* can be correlated adequately with the conditions of its functioning *in vivo*. Such a doubt is quite equitable since it became clear[9-12] that the subcellular structure and the compartmentalization of enzymes play the most important role in metabolism and regulation.

I.2 Enzymes in Biotechnology

The application of enzymes in chemical technology has often been advocated for reasons of high reaction selectivity and stereospecificity. The second major advantage of enzyme technology over conventional chemical catalysis relates to the high catalytic rates possible under relatively mild reaction conditions. The use of enzyme processes in industry[13,14] are well known. Hydrolase, racemase, lyase are used in the preparation of amino acids. The use of proteinases in the food industry has a long history. The most pre-eminent of immobilized enzymes in industrial use is glucose isomerase, catalysing the conversion of

glucose to fructose. In the starch industry the use of enzymes enables the degree of hydrolysis to be controlled to produce a product with the desired physical properties , such as viscosity, sweetness, osmotic pressure and resistance to crystallization.

The future impact of biotechnology upon the chemical industry will rely upon the successful integration of basic microbiology and biochemistry with chemical technology. There are several areas in which a potentially exciting future may be identified.

(1) Reaction type: direct oxidation/oxygenation reactions; use of common enzymes for unexpected chemistry; protein engineering to alter catalyst properties ; use of biological catalysts in non-aqueous environments.

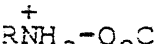
(2) Reactor configuration: optimization of biocatalysts by genetic engineering, selection of thermostable systems or use of immobilized configurations; development of cheap, stable, efficient and generally applicable methods of cofactor recycling; chemical engineering in relation to large scale biocatalytic systems.

I.3 Surfactants: For Preparation of Micelles and Reverse Micelles

To simulate the structure and functions of enzyme containing fragments of the cell that is biological membranes <u>in vitro</u> various media have been proposed. Today, both normal micelles and reverse micelles in non-aqueous media have been recognised to possess sound features similar to biological membranes.[15-17]

I.1 Surfactants which Aggregate in Aqueous and Non-aqueous Solvents

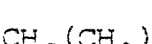
Cationics



Alkylammonium carboxylate

R=$C_{12}H_{25}$, R'=C_2H_5, DAP

R=$C_{12}H_{25}$, R'=C_6H_5, DABz

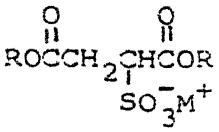


Cetyltrimethylammonium

X=Br, CTAB

usually requires a cosur

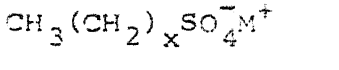
Anionics



sulfosuccinates

R = 2-ethylhexyl

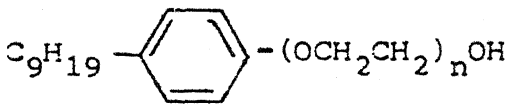
M^+= Na^+; AOT



x = 11, M = Na; SDS

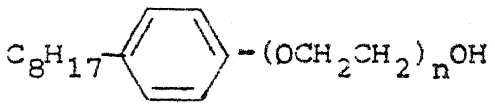
x = 15, M = Na; SHS

Nonionics



Polyoxyethylene nonylphenols

n = 6, Igepal CO-530

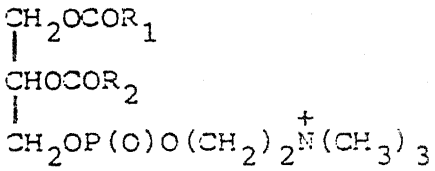


Polyoxyethylene octylphenol

n = 9.5 ; Triton X-100

n = 5 ; Triton X-45

Zwitterionics



Phosphatidylcholines (Lecithin)

Surfactants, surface active agents, or detergents are amphiphilic, organic or organometallic compounds. Amphiphilic substances, or amphiphilies, are molecules possessing distinct regions of hydrophobic (water-repelling) and hydrophilic (lipophilic or water-attracting) character. The surfactant may be classed as cationic, anionic, nonionic, or ampholytic (zwitterionic) depending on the chemical structure of the hydrophilic moiety bound to the hydrophobic portion.

Cationic surfactants have the general formula of $R_nX^+Y^-$, where R represents one or more hydrophobic chains, X is an element capable of forming an "onium" structure, and Y is the counterion. The most frequently used anionic surfactants are alkali and alkaline earthmetal salts of mono- or polybasic caboxylic (fatty) acids and of sulfuric, sulfonic and phosphoric acids containing a saturated or unsaturated hydrocarbon substituent.

Zwitterionic surfactants possess both anionic and cationic groups on the hydrophobic moiety and depending on the pH of the solution and the structure , can behave as either an anionic, cationic, or neutral species. The more common zwitterionic surfactants include N-alkyl and C-alkyl betaines and sultaines as well as phosphatidyl amino alcohols and acids.

Most nonionic surfactants are polyoxyethylene and polyoxypropylene derivatives (of compounds such as alkyl phenols and alcohols, fatty acid esters, and alkylamines, amides, and mercaptans) or polyalcohols, carbohydrate esters, fatty alkanol amides, and fatty amine oxides.

I.3.1 Micelles or Normal Micelles

Surfactants or detergents or surface active agents dynamically associate through hydrophobic interaction in aqueous solution above a certain critical concentration , referred to as critical micelle concentration (CMC) to form large molecular aggregates of colloidal dimensions called micelles. Above the CMC there exists a dynamic equilibrium between surfactant monomers and micelles. However, it has been suggested that an indefinite self-association model of step-wise aggregation may be more realistic.[18]

Each micelle is composed of a certain number of surfactant molecules called aggregation number which decides the general size and geometry of the particular system. The CMC as well as the aggregation number are well known to be sensitive to organic and inorganic additives. At high concentrations of surfactant e.g. more than 20 times the CMC, the micelles enlarge and aggregate, ultimately to produce liquid crystalline phases.[19] Micelle formation is accompanied by well-defined changes in various physical properties like interfacial tension, electrical conductivity, e.m.f., pH, density, specific heat, temperature coefficients of solubility, transport properties such as viscosity, optical and spectroscopic properties of the solution.

I.3.2 Structure of Micelles

Although aqueous micelles have been investigated for more than six decades, detailed understanding of their structures only began to emerge recently.[20] The micellar structure is such that

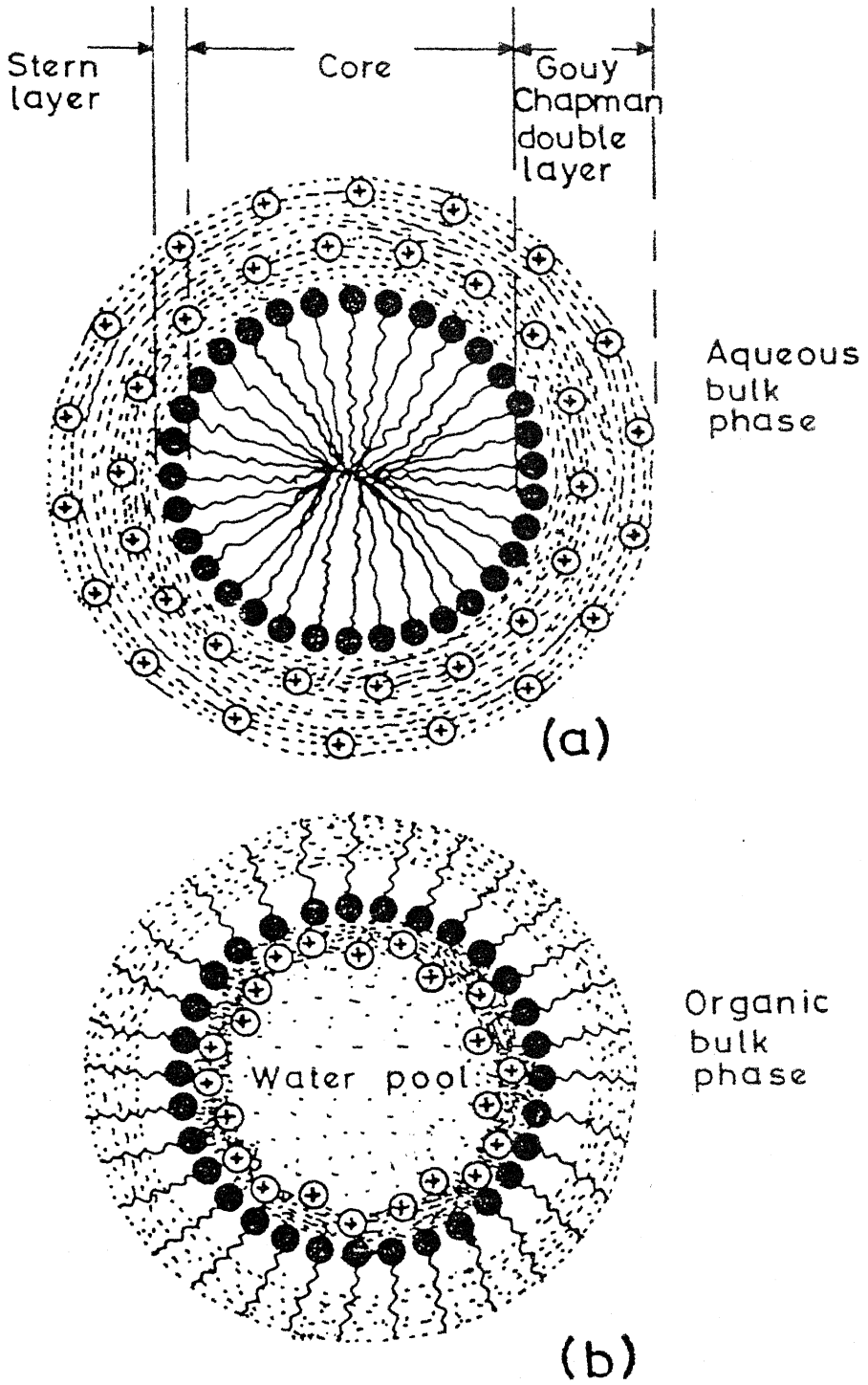


Fig.I.1 Spherical cross-section of an idealized anionic (a) normal micelle (b) reverse micelle. (•) the polar head group; (⊖) the counterion; (∿∿∿) the hydrocarbon chain.

the hydrophilic head groups are directed towards and in contact with the aqueous solution, thus forming a polar surface, while the hydrophobic tails are directed away from the water, forming a central nonpolar liquid-like hydrocarbon micellar core. The polar head groups are at the micellar surface which is rough. A fraction of the counterions is bound to the surface and forms part of the micellar aggregates. The layer containing the polar head groups and counterions is termed as Stern layer. The remaining counterions which are located outside the micellar surface, form the diffused Gouy-Chapman double layer. The counterions in the double layer exchange with ions of similar charge in the bulk solutions.[21] Micelle size, shape and fraction of surface charge that is neutralised, vary with the concentration and nature of the counterion. Though, there have been controversies regarding the penetration of water into the micelle it is now accepted [22] that one or more methylene groups attached to apolar groups are exposed to water. Solvent sensitive ^{13}C NMR chemical shifts of carbonyls inserted into micelles were used to probe the micellar microenvironments.[22] Both the NMR data and the molecular models[20] point to a complex and diverse micellar interior whose polarity covers a wide range.

In concentrated solutions of surfactants a wide variety of structures and mesophases have been identified. Nuclear magnetic resonance may provide evidence on micelle structure and mobility and also on the site of solubilization. The aggregation number of micelles also depends upon ionic strength. For example, the aggregation number of SDS increases from 95 to 117 to 132 as the NaCl concentration is elevated from 0.0 to 0.1 to 0.4.[23] On the

other hand, organic additives generally decrease the aggregation number. Thus, micelles appear to be unfettered structures, held together in a delicate balance of forces and are able to contract or expand depending upon the condition of the surfactant solution. It may be noted here that the kinetic data fit this loose ball picture in which case the monomer remains in a micelle only for 10^{-3} to 10^{-8} sec depending on the chain length of the surfactant.[24]

I.3.3 Dynamics of Micellization

Micelles are dynamic species. A number of independent measurements indicate that the micelles are not static but are in dynamic equlibrium with coexisting monomers and pre-micellar aggregates. They rapidly break up and reform. Kinetics of micelle association and dissociation in surfactant solutions has been studied, and consequently , several models (e.g. multiple equilibrium model, collision model, and co-operative model) have been developed.[25] The micellar aggregates formed by ionic surfactants in dilute aqueous solutions reorganise rapidly on a chemical time-scale. Rate constants derived for such processes depend on the techniques of measurement. There is a fast process, characterised by a relaxation time, which is usually detected by ultrasonics but may also be observed in temperature jump and shock tube experiments.The fast process relaxation time T_1 is treated as a pseudo equilibrium and the slow process relaxation time T_2 as a pseudo stationary flow, following small perturbations. Depending upon the chain length of the surfactant, T_1 is in the range of 10^{-3} - 10^{-8}s and T_2 in the

range of 10^{-1} - 10^{-4}s. In the fast process of the exchange of monomer between micelle and bulk solution, reorganization of the counterion being assumed to be undetectably fast. The slow process is the total dissociation of micelle to monomers. In concentrated surfactant solutions there is evidence for second equilibrium, perhaps the transformation of spherical into rod-shaped micelles.[26]

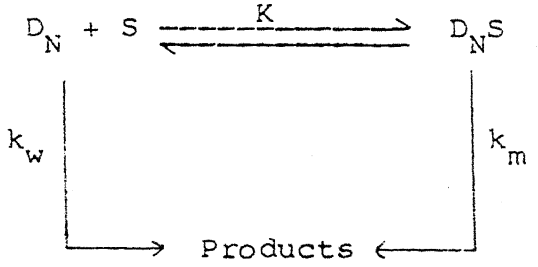
I.3.4 Decrease of Free Energy in Micellization

The thermodynamics of micellization have been treated using several models. The decrease in the overall free energy of the system which results from the preferential self-association of the hydrophobic hydrocarbon chains of monomeric surfactant molucules is the driving force for the formation of micellar aggregates. The mass-action and phase-separation models are the simplest and most frequently used treatments. The former applies the law of mass action between monomeric surfactant, with counterions, and micelles. The alternative phase separation model considers micelles, together with their counterions, as a separate phase that appears at the CMC. Both the mass-action and phase-separation models are gross oversimplifications. Different workers[27-33] show that micellar effects could be observed well below the critical micelle concentration which is attributed to pre-micellar association, i.e, the formation of small aggregates with much less number of surfactant monomers than the aggregation number of surfactant. The free energy changes which occur with increasing surfactant concentration are also manifestations of the change in water structure and, hence, changes in the entropy

of the system. The free energy of micelle formation has been found to be more dependent on entropy than on enthalpy factors.

I.3.5 Models to Analyse Micellar Catalysis

To rationalise the general features of micellar catalysis quantitatively, efforts have been made and consequently a number of models have been proposed.[34-38] The quantitative treatment of the kinetic effects of micelles, developed by Menger and Portnoy[34] to describe the relationship of rate constant to surfactant concentration, assumes that the micelle (D_N) forms a noncovalent complex with substrate (S), which may then react to yield product as shown below in scheme I.1.



Scheme I.1

Here K is the association constant of the micelle-substrate complex; k_w and k_m are the first order rate constants for the reaction in the aqueous and micellar pseudophases respectively. The observed first order rate constant , k_ψ , at any concentration of micelle, according to Scheme I.1 is given by

$$k_\psi = \frac{k_w + k_m K C_M}{1 + K C_M} \qquad \ldots \quad I.1$$

where C_M is the concentration of micelles and is given by

$$C_M = \frac{C_D - CMC}{N} \qquad \ldots \text{ I.2}$$

in which C_D is the concentration of surfactant, CMC is the critical micelle concentration and N is the aggregation number of the micelle. Combination of Equations(I.1) and (I.2) and rearrangement yields the following equation

$$\frac{1}{k_w - k_\psi} = \frac{1}{k_w - k_m} + \frac{N}{K(C_D - CMC)} \qquad \ldots \text{ I.3}$$

Equation(I.3) describes the dependence of the rates on the surfactant concentration for many micelle catalysed reactions. It is to be noted here that a similiar equation (Lineweaver-Burk Equation) is obtained for enzyme catalysed reactions following Michaelis-Menten saturation kinetics. Applicability of this model is limited to micellar catalysed and inhibited unimolecular and micellar inhibited bimolecular reactions where rate constant increases with increasing surfactant concentration to a satturation value.

I.3.6 Biological Importance of Surfactant Micelle System

Micelles are assumed to provide microenvironment somewhat similar to that of active binding site of enzyme. This assumption is based on the following observations:

(a) X-ray crystallographic study has indicated the similarity between micelles and globular proteins

(b) Both micelles and enzymes bind substrate in non-covalent fashion; and

(c) Resemblance between enzyme and micelle catalysed reactions.

A large number of biologically important reactions such as dephosphorylation, deacylation, decomposition of diazotates, hydrolysis of acetals and ortho-esters, etc. have been studied in the presence of micelles to improve our understanding of enzymatic systems. Importance of micelles was further increased when Nagyvary and Fendler[39-43] implicated micelles in the origin of life. These authors have suggested that micelles present at primitive ocean surface might have provided appropriate environment for selective uptake of amino acids and thus might have catalysed the formation of polyncleotides and polypeptides. Photoionization of the molecule together with the subsequent electron transfer reactions as well as photoredox reactions in which electron is transferred from low energy acceptor using visible radiation, are also being studied in presence of micelles because of their importance in solar energy conversion, storage and their relevance to biological electron transfer processes.[44-47]

I.4 Reverse Micelles

Molecular association is not limited to aqueous solutions. Formation of association colloids can also occur in various

nonpolar solvents in which case the surfactant aggregates are termed reverse micelles.[17,24,48-53] In reverse micellar systems, the polar head groups of the amphiphiles are concentrated in the interior of the aggregates and hence form a central hydrophilic polar core. The hydrophobic hydrocarbon chains extend into and are in contact with the bulk nonpolar solvent. The size and shape of reverse micellar systems in nonpolar solvents vary considerably and depend upon factors such as surfactant concentration, surfactant structure, absence or presence of added solutes, and the nature of the nonpolar solvent employed. Reverse micelles possess low degree of aggregation and the aggregational properties of surfactants in nonpolar media are often altered markedly by the traces of water. Surfactant association in apolar solvents is predominantly the consequence of dipole-dipole and ion pair interactions between amphiphiles. This is quite different from the opposing hydrophobic attractions-electrostatic repulsions responsible for micellization in water.

I.4.1 Structure of Reverse Micelle

The micellization process has been treated as a pseudophase model or a multiple equlibrium phenomenon.[54-57] Unlike surfactant aggregation in aqueous solutions, which is often characterised by a well-defined critical micelle concentration (CMC) and monomer $\longleftrightarrow$ $\bar{n}$-mer association, surfactants in nonpolar solvents often display indefinite self association.[58-63] In several cases the step-wise equlibrium constants for the aggregation monomer $\longleftrightarrow$ dimer $\longleftrightarrow$ trimer $\longleftrightarrow$ $\bar{n}$-mer have been found to be equal; average aggregation numbers are

frequently as low as three to seven at moderate total surfactant concentrations. The sequential indefinite type self association is described by

$$S_1 + S_1 \underset{}{\overset{K_2}{\rightleftharpoons}} S_2 \qquad K_2 = \frac{[S_2]}{[S_1]^2}$$

$$S_1 + S_2 \overset{K_3}{\rightleftharpoons} S_3 \qquad K_3 = \frac{[S_3]}{[S_1][S_2]}$$

$$\vdots \qquad\qquad \vdots \qquad\qquad \vdots$$

$$S_1 + S_{n-1} \rightleftharpoons S_n \qquad K_n = \frac{[S_n]}{[S_1][S_{n-1}]}$$

On the molar concentration scale,

$$[S_2] = K_2[S_1]^2$$

$$[S_3] = K_2K_3[S_1]^3$$

$$\vdots$$

$$[S_n] = K_2K_3 \ldots\ldots\ldots K_n[S_1]^n$$

and, if all equilibrium constants are assumed to be equal (i.e., $K_2=K_3\ldots\ldots K_n$), the weight fraction of the monomer, f_1, can be shown to be related to the molecular weight of the monomeric surfactant, M_1, and to the stoichiometric surfactant concentration, C_o expressed in the gm^{-1} scale, by

$$\frac{1-f_1^{1/2}}{f_1} = \frac{1000K_2}{M_1} C_o$$

Mono-, di-, and trialkylammonium salts and many nonionic surfactants display this aggregation behavior.

There are also surfactants which display monomer $\longleftrightarrow$ $\bar{n}$-mer association in nonpolar solvents and they often contain two

hydrocarbon chains. This is called phase transition model. Dialkyl sulfosuccinates[52,64-67] of which AOT is an example, dinonylnaphthalene sulfonates,[68] and phosphatidylcholines[69-71] are perhaps the most extensively studied. Aggregation numbers typically range from 12 to about 30, but dodecylammonium benzoate forms a trimer in a monomer $\longleftrightarrow$ $\bar{n}$-mer association.[72] Even with these surfactants, the definition of a CMC is not without ambiguity.

In cases where CMCs have been measured for surfactant aggregation in apolar solvents, the values are frequently in millimolar range. The smallest AOT aggregates[65,73] observed in isooctane (2,2,4-trimethylpentane) have a hydrodynamic radius of 15 Å which is longer than the extended length of the surfactant molecule (ca. 11 to 12 Å). For the "dry" AOT micelles, the aggregation number shows a large dependence on the hydrocarbon solvent used.[74,75] The micellar size increases as the ability of the hydrocarbon solvent to penetrate into the curved AOT monolayer decreases.

I.4.2 Structural and Thermodynamic Reason for Interaction Between Reverse Micelles

Several factors emerge as contributing to the thermodynamic behavior of a reverse miccellar system and the stability of the single-phase system. Taking AOT as an example, at the low temperature limit, the AOT ceases to be effective as a dispersant. At and above the high temperature limit, the surfactant has less affinity for the continuous oil phase. For a

particular tempreture, the extent to which this occurs depends on the oil used as the dispersion medium. There is 'clustering' of micelles in the vicinity of the upper temperature phase transition, which in concentrated microemulsions can be understood in terms of percolation . Associated with percolation are changes in electrical conductivity which are ascribed to mechanisms involving fusion of aggregates or surfactant transport along the cluster network. The onset of such percolation effects can occur over a range of dispersed phase volume fractions, depending on the oil and temperature.

The instability of the reverse micellar system does, however, have some distinct advantages in synthesis: the reversible nature of the phase transitions and the mild conditions under which such phase transitions are induced suggest a convenient method of separation of products for reactions which have taken place in the one-phase reverse micellar system.

I.4.3 Dynamic Aspects for Interaction Between Reverse Micelles

It was demonstrated first by Eicke et al.that exchange of material between reverse micelle was facile.[76] A number of studies have indicated that while exchange of small solubilizate molecules among reverse micelles is generally much more rapid than any chemical reactions in which the molecules may participate (this may not be true for enzymes as solubilizates), exchange is a factor of ca. 10^3 below the diffusion-controlled rate.[77,78] Fig.I-2 depicts two possibilites for the exchange of water pool contents. Mechanism I involves the transient fusion of

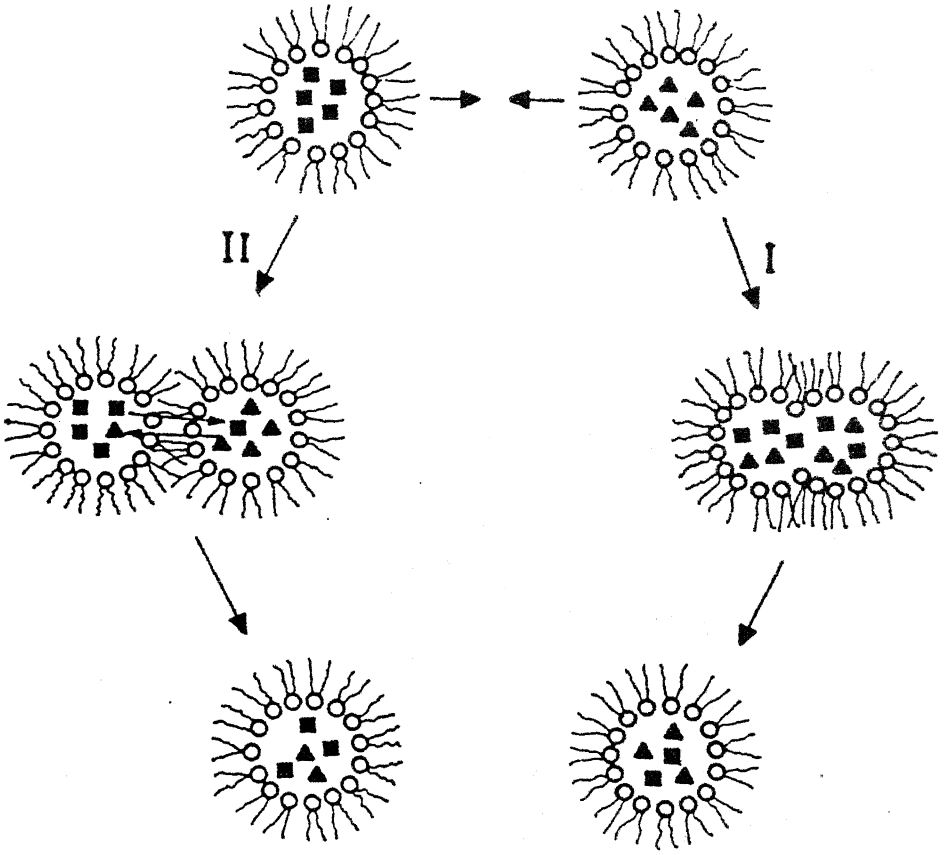


Fig.I.2 Possibilities for the exchange process of guest molecules in reverse micelles: (I) diffusion of the exchanging species with formation of a transient dimer; (II) exchange is mediated with maintenance of water pool integrity.

two micelles to form a short-lived 'dimer droplet.' During the finite lifetime of this dimer species, solubilized species (together with water and surfactant) can randomly redistribute by translational diffusion. The discrete nature of the micellar dispersion will depend on both the fusion rate constant and the life time of the dimer. The faster the fusion rate constant and the longer the life time of the fused species (dimer), the less discrete the dispersion will be. The fission process is not expected to be exact, in the sense that the reformed micelles are identical, and micelles of different size may be predicted on statistical grounds, providing a kinetic insight into the poly-dispersity which undoubtedly exists in these systems .

Finally, the process of fusion is probably associated with the release of some surfactant into the oil phase, since the interfacial area/volume ratio would decrease on fusion; this would provide a possible explanation for the small amount of surfactant which apparently co-exists in the form of 'nearly-dry' reverse micelles in these systems.[79]

The idea for mechanism II is the diffusion of the exchanging species through the surfactant bilayer formed at the point of contact of non-fusing micelles. If this mechanism were to operate, it might be expected that the rate of solubilizate transfer through the bilayer would be reflected in k_{ex} values which are very dependent on the size and charge of the ion being transferred. This is not found experimentally, providing strong support for mechanism I as the preferred mechanism.

I.4.4 The properties of water in water pool of Reverse Micelle

Water localized in the interior of reverse micelles differs from that of bulk water. The anomalous water at low w_o will obviously influence the chemical behavior of guest molecules. The endomicellar water pool may be thought of as a series of regions in which the water molecules are characterized by different correlation times, which depend on the geometrical constraints to which they are exposed (cage effects) and on their dipolar interactions with one another and with the polar groups of the micellar wall. The size of and also shape of reverse micelles are critically dependent on the number of water molecules available per molar head of the surfactant (w_o). The AOT/H_2O/alkane system has been investigated by the electrical birefringence technique in the presence and absence of electrolyte.[80,81] As already discussed, the size of the micelles changes with the amount of water added, and their shape has been found to change from ellipsoidal to more spherical when electrolyte is added.

The state of the water in AOT reverse micelles in heptane has been investigated by NMR spectroscopy[82] over a large w_o range ($1 < w_o < 50$): water is highly immobilized in the micellar interior at low w_o and the mobility increases with increasing w_o, gradually approaching that of bulk water.

Water solubilized in apolar solvents by different surfactants (cationic, anionic and zwitterionic) exhibits two absorption bands in the near infrared spectral region (5200-4700 cm^{-1}).[83,84] This finding has been interpreted as due to two water

populations, one bound to the surfactant polar head and the other dispersed in the bulk phase.

I.4.5 Distribution of Guest Molecules in Reverse Micelles

To explain the distribution mode of probes in aqueous and reverse micelles[85-90] several models have been proposed. The various schemes are as follows:

1. Random distribution: solubilization of a probe does not perturb the micellar structure and solubilization of additional probes is on a random basis (Poisson).[85-91]

2. Co-operative (attractive) distribution: this implies that the probes are preferentially solubilized in micelles which already contain a probe.

3. Repulsive distribution: this implies preferential solubilization of probes in empty micelles.

In both aqueous and reverse micelles, the distribution law is deduced from the study of the mechanism of quenching of a sensitizer by either a guest molecule or from exciplex formation.

The probability of finding j quenchers per micelle, P_j, is given by

$$P_j = \frac{[M_j]}{[M]}$$

where $[M_j]$ and [M] are the concentration of micelles containing j quenchers and the total micellar concentration, respectively.

The average number of quenchers per micelle is given by

$$n = \frac{[Q]}{[M]}$$

where [Q] is the quencher concentration. P_j is directly related to the distribution law of quenchers. It has been shown that for a random distribution (or Poisson distribution)

$$P_j = \frac{n^j e^{-n}}{j!}$$

whereas for a particular repulsive distribution an appropriate equation would be

$$P_j = \frac{n^j . m!}{m^m (m-n)^j - mj! (m-j)!}$$

where m is the maximum permitted number of quenchers in the micelle.

I.4.6 Reverse Micelles as Novel Microenvironments for Chemical Reactions

Investigations of reverse micellar media can provide significant information relevant to enzymatic and cell membrane interactions, organic and inorganic reaction mechanisms, and small and large scale industrial processes. Functional groups of reverse micelles are capable of binding substrates fairly strongly in specific orientations and configurations.

It is possible to solubilize controlled amounts of substrates and water separately and concomitantly in the cavities of reverse micelles. Under such conditions it is quite feasible to investigate reactions kinetically as functions of both substrate and water concentration. In aqueous solution where water is both a reactant and a reaction medium, direct determination of the role of water in the rate-determining step is not possible. Additionally, the nature of the cavity can be easily altered to investigate effects of (i) the size of the cavity (ii) its polarity and structure of the polar functional groups, (iii) its water content, and (iv) coordinately bonded and "free" metal ions on both substrate interactions and catalysis.

Variety of chemical reactions (ester hydrolysis and aminolysis, metal ion-ligand exchange, proton transfer, etc) have been studied in reverse micelles. It has been established that rates in the presence of micelles are greater than in either the bulk organic solvents or bulk water.[48,58]

The mutarotation of 2,3,4,6-tetramethyl-α-D-glucose is not catalyzed either by aqueous micelles or by bifunctional or micelle-induced bifunctional catalysts. Very substantial rate enhancements are observed, however, in benzene and in cyclohexane by micellar dodecylammonium propionate, butanoate, and benzoate. The rate enhancement by DAP in cyclohexane is 863 fold. The greater rate enhancement in this latter solvent is the consequence of more favourable partitioning between the polar micellar phase and the bulk apolar solvent in cyclohexane than in benzene.

The rate constant for the decomposition of sodium-1,1-dimethoxy-2,4,6-trinitrocyclohexadienylide in benzene in the presence of dodecylammonium benzoate, containing 0.05% DMSO (v/v), is greater by factors of 62,900 and 1800 than that in pure benzene or in pure water.[92] Decomposition of the methoxyl adduct of 1-methoxy-2,4-dinitronaphthalene is catalyzed to an even greater extent by dodecylammonium benzoate in benzene. Under the conditions of the experiment, the reaction is so rapid, that its kinetic rate constant could not be obtained. Decomposition of the methoxyl adduct of 1-methoxy-2,4,6-trinitrobenzene follows saturation kinetics both with respect to the substrate and with respect to the surfactant. In this regard, catalysis by reverse micelles in non-aqueous solvents resembles enzymatic and micellar catalysis in aqueous solvents.

Both reverse micellar catalysis and the roles of solubilized water in reverse micellar systems have been examined for aquation, electron transfer, and isomerization reactions.[93-95]

I.4.7 Technological Relevance of Reverse Micelles

Most of the applications of reverse micelles are based upon their ability to solubilize substances. They can also be used as a 'reservoir' of monomers or to play special roles in absorption processes. These properties have been exploited in the field of oil recovery, lubrication, detergents and catalysis, etc.[96-100]

I.4.8 Photochemistry in Reverse Micelles

Reverse micelles can solubilize proteins. Photoelectron

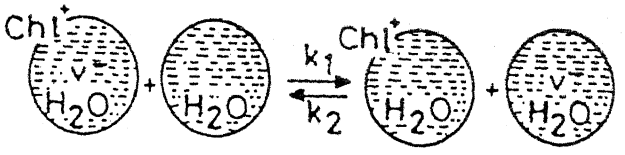
transfer reactions with photosynthetic proteins can be studied and the system may be considered to be a reasonably good model of thylakoid membranes. The exchange processes occurring in reverse micelles can induce charge separation between the photolytic products formed by electron transfer. In photoelectron transfer reactions there are two main components, protosensitizer (D) and electron acceptor (A).

The reaction is as follows

$$D + h\nu \longrightarrow D^*$$

$$D^* + A \longrightarrow D^+ + A^-$$

The role of microemulsions is to perform the functions of the thylakoid membrane. Judicious organization of D and A should bring about favourable energy deposition and transmission and importantly, prevent back electron transfer between A^- and D^+. With the sensitizer located at the micellar interface and the acceptor in the water pool, photolytic products can be separated using reverse micelle. A kinetic model has been proposed for the reaction of chlorophyll as sensitiser and viologen as acceptor.



Non-aqueous bulk solvent

Chl^+ = Chlorophyll
v^- = viologen

Scheme I.2

I.5 Enzymes in Reverse Micelle

The enzymatic reactions in the living cell often proceed in close proximity to the interface. Naturally the question is raised whether the enzyme properties observed in vitro can be correlated adequately with the conditions of its functioning in the living cell. One of the possible ways to do this is to carry out investigations on enzymes in model systems. Reverse micelles in organic solvent can be used as such a model system. It is known that water soluble enzymes may be solubilized in organic solvents in presence of surfactants, with the retention of catalylic activity and specificity of the enzymes. The enzyme on solubilization is entrapped into the inner cavity of reverse micelle and is thus protected from the denaturing effect of the organic solvent. The water inside reverse micelles differs notably in its properties from the aqueous macrophase.[58,101-104] In the colloidal system the enzyme molecule is located near the interface. This approach (the dissolution of an enzyme in the colloidal solution of water in an organic solvent) apparently offers a realistic model for enzymological studies in vitro.

I.5.1 Solubilization of Enzymes in Reverse Micellar Solutions

Several enzymes have been solubilized in reverse micellar solutions. In fact solubilization of enzymes in reverse micelle is superior to the alternative procedure for utilizing enzymes in organic solvents, such as biphasic systems[105] or suspensions of enzymes in organic solvents.[106] The study of enzymes hosted in reverse micelles can be carried out by utilising the same

techniques as for aqueous solutions. The micellar solutions are transparent and amenable to all kinds of spectroscopic studies, e.g., absorption, fluorescence, NMR, as well as other methods like ultraviolet transient kinetics, relaxation techniques, calorimetry, etc. The clear solution by solubilization of enzymes in reverse micelle enables the study of enzyme mechanisms under controlled and reproducible conditions, which is another considerable advantage in comparison to the alternative media (biphasic systems or suspensions).

I.5.2 Structure of Enzymes in Reverse Micelles

A very pertinent question regarding the solubilization of enzymes or biopolymers is about the location of biopolymers in the reverse micelle. The structure of the water, counterion of the surfactant in the interior of the reverse micelle is also important.

The most acceptable model is the so called "water-shell model". According to this model the enzyme is confined to the middle of the water pool and is protected by a layer of water from the charged inner wall of the micelle. The evidence for this model is still indirect. For example, the conformation and activity of enzymes depend on w_o which indicates that the enzyme is sensitive to the amount of water surrounding it. This would not be so if the enzyme adhered with its active site region to the internal wall of the reverse micelle, or if the enzyme were predominantly exposed to the hydrocarbon solvent.

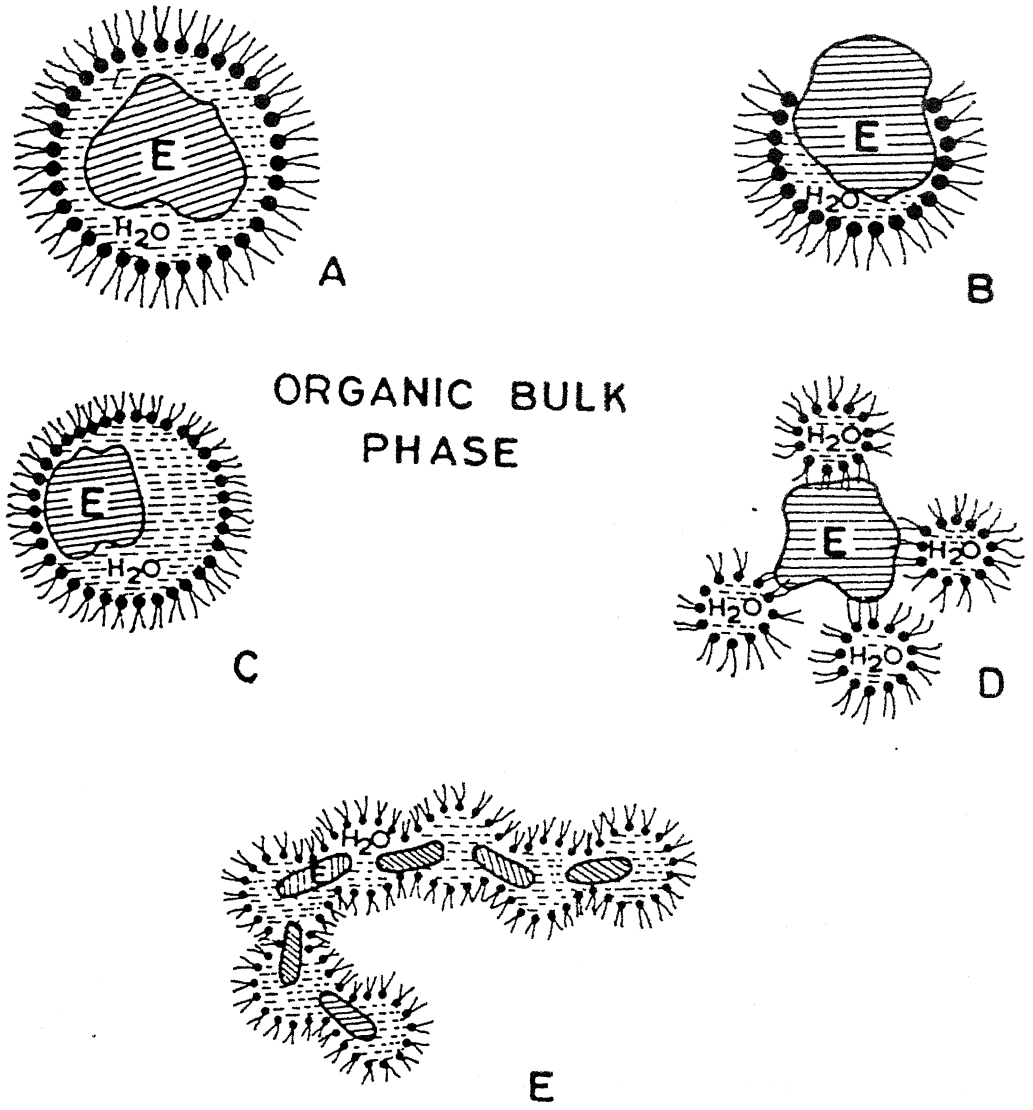


Fig.I.3 Possible models for an enzyme hosted in a reverse micelle. (A) the water-shell model whereby the enzyme located in the water pool and separated from the micelle wall by water layers; (B) the case of an enzyme having a very lipophilic part; (C) enzyme adsorbed to the micelle wall; (D) enzyme solubilized by the help of several small micelles; (E) formation of a network among several micelles, bridged by the enzyme molecules.

In principle, other modes of hosting a biopolymer inside reverse micelles can be envisaged, particularly if the protein is not very hydrophilic. A few possible situations are presented in Fig. I-3. It is quite conceivable that a highly hydrophobic protein may tend to interact with the hydrocarbon chains of the surfactant or even with the organic solvent. A model in which the protein partly adheres to the charged internal wall of the micelle is very attractive in view of the analogy with enzymes bound to insoluble matrices.

I.5.3 Spectral Characteristics of Enzymes Incorporated in Reverse Micellar Water Pool

In reverse micelles there is a general tendency towards a red shift for UV absorption spectra when compared to that in water solution. This can be explained on the basis of less polar character of the water pool environment. Changes in the extinction coefficients in micelles with respect to water are absent or modest.

At high water pool the fluorescence spectra in water and in reverse micelles are very close to each other. Only at very small w_o values there is, at least in the AOT system, a sizable enhancement of the quantum yield and blue-shift of the emission spectra. A systematic investigation of fluorescence polarization could be useful to determine the relative mobility of the guest protein. The mobility is also related to the dynamics and exchange processes of protein-containing reverse micelles.

In CD spectra the general trend is similar to that observed with UV absorption and fluorescence, in as much as perturbations with respect to water solution are larger, the smaller the w_o, and tend to disappear at larger water contents. In addition, there is an increase of ellipticity in the far UV- region of the spectrum, namely, in correspondence with the electronic transitions of the peptide chromophore. This can be taken to suggest an increase of secondary (in general helical) structure. This is in keeping with the notion of a more hydrophobic environment: hydrogen bonding tends obviously to increase in a solvent less polar than water.

I.5.4 Reverse Micelles as Suggested Model of Biological Membranes for Enzymes

The hypothesis that reverse micelles constitute a realistic model of biological membrane structures has been frequently stated in the literature.[42,84,103] It is known that the bilayer membranes frequently contain nonbilayer structures, the so called lipid particles. Investigations showed that these particles consist of lipid molecules associated in the form of reverse micelles and incorporated between the monolayers of the bilayer membrane (Fig.I-4a). The most important cell processes such as the coalescence and compartmentalisation of the membranes,[107-113] exocytosis,[108] the transport of lipids through membranes via the "flip-flop" mechanism,[114,115] and the transmembrane transport of ions,[108] proceed via a mechanism similar to that[114] illustrated in Fig.I-4b for protein molecules. Certain enzymes are capable of inducing the formation of reverse

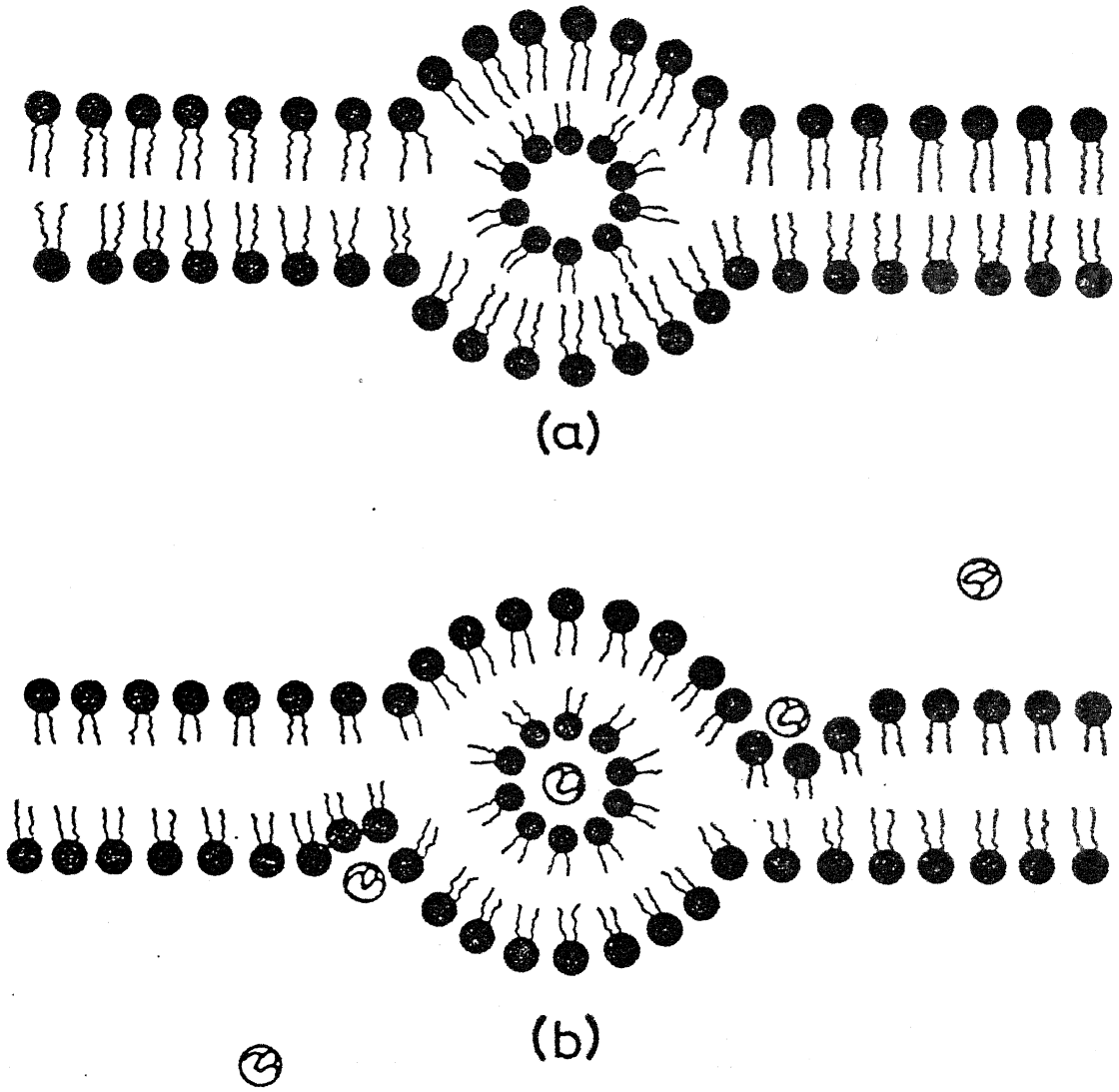


Fig.I.4 A possible function of the intramembrane lipid particle (a) biological membrane; (b) transfer of a protein molecule through membrane.

micelles in a bilayer membrane, while being incorporated at the same time in the inner cavity of such a micelle. It is possible that the regulatory effects observed in the study of characteristic features of the equilibrium of an enzyme reaction in a micellar system occur also in a living cell where the content of metabolites might be regulated by varying the degree of hydration of biological membranes.

I.5.5 Catalytic Activity of Enzymes Solubilised in Reverse Micellar Solution

Several enzymes have been solubilized in reverse micellar solutions and found to be active. Major thrust of these investigations have been directed towards (a) the elucidation of the structure of enzyme-containing micelles and the enzymology of these novel catalytic systems, and (b) the applications of micellar enzymology to biotechnology. The activity and behaviour of enzymes in reverse micellar system can be described by the following generalizations (i) Solubilized enzymes are able to maintain activity comparable to that found in aqueous solutions; (ii) Any significant changes in the kinetic behavior, have not been reported so far, (iii) The maximal activity in reverse micelles is not found at the maximal water content (e.g. at w_o around 50 to 70), but at rather lower w_o values, (iv) Enzymes with substrates sparingly soluble in water can be studied under conditions of substrate saturation without any difficulty of solubility.

I.5.6 Effect of the Degree of Hydration on Activity of Enzymes in Reverse Micelles

The degree of hydration i.e. w_o is an important parameter in micellar enzymology. It determines the size and properties of the micro-medium within the inner cavity of the micelles. The dependence of the catalytic activity of the solubilised enzymes on the degree of hydration of the reverse micelle has been investigated for enzymes such as α-chymotrypsin,[113,116-118] trypsin,[119] lysozyme,[120] phospholipase A_2,[121] alcohol dehydrogenase,[116] pyrophosphatase,[116,118,122] malate dehydrogenase[123], glutathione reductase[124], lactate dehydrogenase[125] and pancreatic lipase.[126] This dependence is bell shaped, i.e. there is an optimum value of w_o for which the catalytic activity of the solubilised enzyme is maximum. The optimum values of w_o differ for different enzymes. The optimum w_o seems to be dependent on a number of factors which either compensate or supplement each other.

I.5.7 Influence of pH on Activity of Enzymes in Reverse Micelle

The rates of enzymic reactions and kinetic parameters describing them are largely pH dependent. In reverse micellar solution marked shift in pH profiles are noted compared to aqueous buffer solution.[127] This is for different reasons. The ionogenic surfactants form a charged electrical layer around the enzyme molecule which can lead to a local shift of the pH. The apparent shift of the pH is usually 1-2 units. The

microenvironment inside the reverse micelle changes the acid-base properties of the ionogenic groups of the solubilised enzymes. Moreover the pK of the ionic compounds may change by several units due to dehydration of ionic groups. Finally, there is a possibility of conformational change in the enzyme on solubilisation, which can also alter the observed pK of its ionogenic groups, including the functional groups involved in the enzyme reaction.

I.5.8 Specificity of Enzymes Solubilised in Reverse Micelle

As a result of its solubilisation in organic solvents change in substrate specificity of some enzymes has been observed. In oxidation of aliphatic alcohols catalysed by horse liver alcohol dehydrogenase in the system of AOT reverse micelles in octane it has been found that the transition from aqueous solution to the reverse micellar medium causes a change in the specificity of the enzyme. Octanol is better substrate in aqueous medium whereas butanol becomes a better substrate in micellar media. A change in the true substrate specificity has been established for pancreatic lipase, which catalyses the hydrolysis of the glycerol esters of aliphatic acids.[126] The transition from an aqueous solution to the reverse micelle system is accompanied by a significant shift of the true substrate specificity of the lipase in favour of high molecular weight substrates.

I.6 Prospects and Applications of Micellar Enzymology

I.6.1 Understanding the Role of Water in Enzymatic Catalysis

Finding out the effect and reaction order with respect to

water in enzymatic reaction in aqueous solution is difficult. On the contrary the amount of water inside the reverse micelle can be varied. It is possible that the research into catalytic properties of enzymes solubilized in organic solvents under different conditions of hydration will enable one to estimate the role of water, not only in the protein structure, but also in the mechanism of the enzymatic catalysis.

I.6.2 Enzymes in Organic Synthesis

In recent years attempts have been directed to look for a medium in which water insoluble or poorly water-soluble compounds can be enzymatically biotransformed conveniently. Such a medium should be at least the following attributes: (i) the solubility of the substrates and cofactors should be high, (ii) the enzyme should maintain high activity, (iii) the product should be easy to recover, (iv) the mass transfer of the reactants to and from the biocatalyst should not be rate limiting. A medium that meets most of these specifications is a medium containing reverse micelles. Water-insoluble or poorly soluble compounds such as steroids, prostaglandins, alkaloids, lipids, fats, etc. can be subjected to a biocatalytic transformations in fine organic synthesis. Veeger and coworkers[128] succeeded in stereospecific synthesis of steroids using three enzymes in reverse micelles. Hydrogenase, lipoamide dehydrogenase and steroid dehydrogenase were solubilised in reverse micelle to specifically reduce a water insoluble ketosteroid to its corresponding hydroxy forms using gaseous hydrogen (Fig.I.5a).

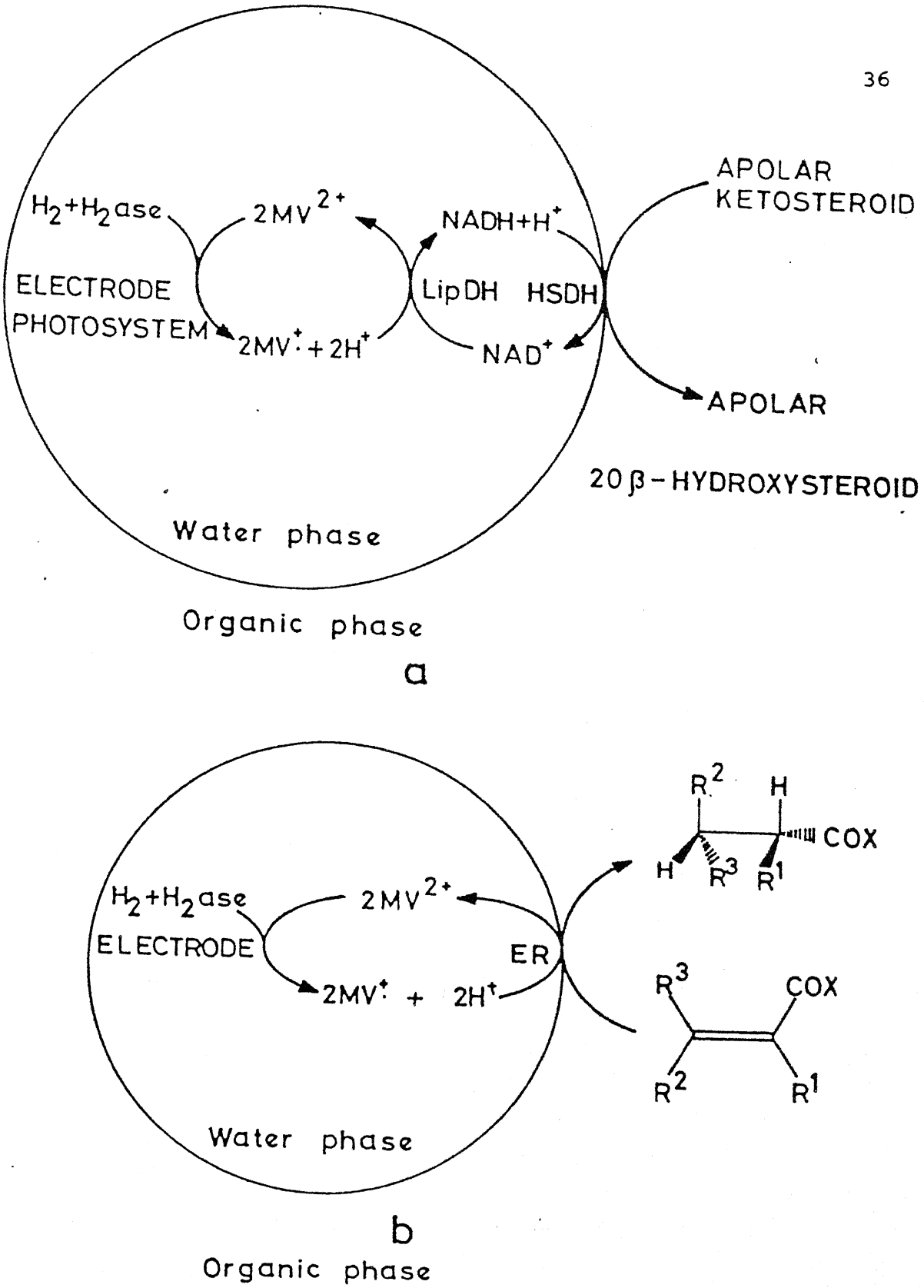


Fig.I.5 Biochemical reactions in reverse micelles. (a) Indirect NADH-mediated reduction of 20-ketosteroids; (b) viologen-mediated reduction of enoates.

The product can be isolated batchwise by transferring the enzymes from the reverse micellar medium to an aqueous medium followed by precipitation of the surfactants and evaporation of organic solvent.

Again C. Laane and his coworkers[129] have shown that employing enol reductase the chiral hydrogenation of α,β-unsaturated carboxylates in reverse micelle is possible (Fig.I·5b).

I.6.3 Enzymatic Synthesis of Peptides with Reverse Micelles

Luisi and coworkers[130] used reverse micelles for the enzymatic synthesis of peptides. They pointed out the potentialities of reverse micelles in enzymatic reactions involving lipophilic reagents. For example a hydrocarbon soluble tripeptide can be synthesised using α-chymotrypsin enzyme.

$$\underset{A}{\text{Z-Ala-Phe-OMe}} + \underset{B}{\text{H-Leu-NH}_2} \xrightleftharpoons{E} \underset{C}{\text{Z-Ala-Phe-Leu-NH}_2} + \text{MeOH}$$

Z is the benzyloxycarbonyl protecting group. Both A and C are practically insoluble in water and soluble in isooctane and therefore the situation can be depicted as in Fig.I·6b.

In the following example only product is insoluble in water.

$$\underset{A}{\text{Ac-Phe-OEt}} + \underset{B}{\text{H-Leu-NH}_2} \rightleftharpoons \underset{C}{\text{Ac-Phe-Leu-NH}_2} + \text{EtOH}$$

Here reverse micelles can be viewed as microreactors, whose dimensions can be easily changed and with a milieu whose physical properties (e.g. dielectric constant, microviscosity, acidity)

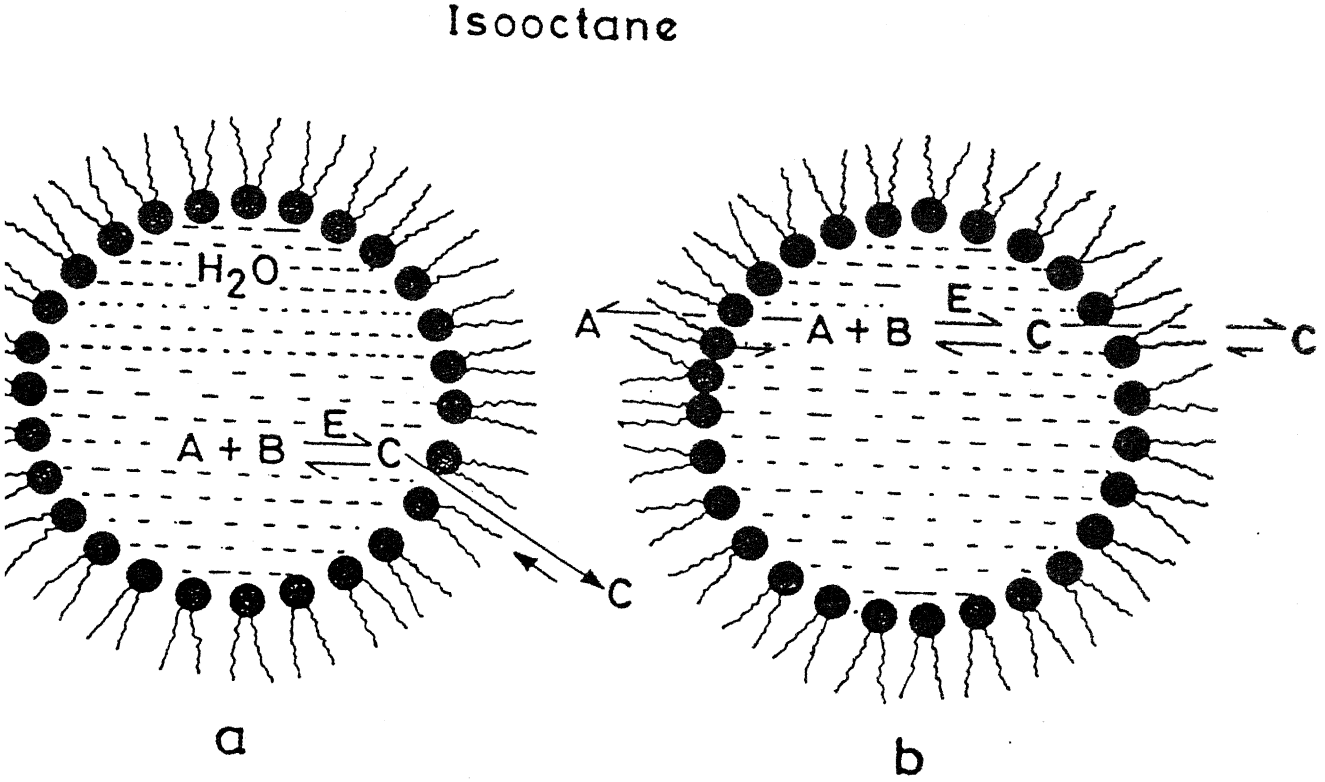


Fig.I.6 Compartmentalization of reactants in reverse micelles in enzymatic reactions. Case (a) the two reagents A and B are preferentially soluble in water and the product C in hydrocarbon; (b) one of the two reagents (A) is also soluble in hydrocarbon.

can be continuously modulated and possibly tailored to the characteristics of the reaction taking place in the water pool. A striking feature of the reverse micellar micro-reactor is the difference between the polar core and the outside (essentially hydrocarbon). This difference in environment can be utilized advantageously for mass transport and separation in chemical reactions.

I.6.4 Nanocapsules of Reverse Micelles as Drug Delivery System

Pharmocologists have been looking for a system which is capable for efficient drug delivery to the target. Liposomes have been extensively used as potentical drug carriers. Speiser[131] et al. have been developing the idea of incorporating drugs into reverse micelles. The water pool of the reverse micelles hosts, together with the drug, olefinic monomers which insert themselves among the surfactant molecules. The olefinic monomers readily polymerise (acrylamide) to a thin resistant skin around the micelle and thus form nanocapsules. After the "hardening" has taken place, the apolar solvent is eliminated, the excess surfactant and monomer washed out, and the nanocapsules are then ready to begin their function in aqueous media. These drug carriers have many pharmaceutical uses. They have been used to study the adjuvant effect in immunobiology of human immunogammaglobulin (IgG), tetanus toxoid and influenza antigen. Also promising is the study of nanocapsules containing active drug molecules, with the aim to bring them directly into the cell interior. The use of endocytosable and lysosomotropic drugs is

promising in connection with the therapy of cancer,[132] intracellular infection[133] and other pathological processes.[134] In reverse micelles, the compartmentalization of drugs in the water pool is obligatory since it is not possible for the hydrophilic drugs to remain in the apolar phase. The method appears promising also for mixtures of hydrophilic drugs in reverse micelles, they will be obliged to compartmentalize in the water pools, even if they do not have great chemical affinity for each other.

I.6.5 Gelation of Reverse Micelles

One of the recent finding in this field is the gelation of reverse micellar solution. Gelatin is used as a matrix and hosted together with the drug in the water pool of reverse micelles. Gelatin is then crosslinked, and the drug should remain entrapped in the net. After removal of the surfactant layer and apolar solvent, one practically has a globular protein which contains the drug in its interior. This "nanopellet" is bicompatible and biodegradable without toxic effects. They are water soluble after complete removal of the micellar components and can be prepared in different sizes. Enzymes can be cosolubilized with gelatin and remain active in gel form. These systems are potentially very interesting for pharmaceutical and cosmetic applications.

I.6.6 Interaction of Antigen and Antibody in Reverse Micelles

In a recent finding it was reported that antigen and

antibody can be successfully solubilized in the reverse micellar water pool. The alterations in the catalytic activity of the enzyme after its interaction with antibodies against the same enzyme have been studied in reverse micellar solution. The enzyme interacted with antibodies very rapidly i.e. the micelles did not hinder effective interaction between the enzyme and antibodies. The decrease in the enzyme activity upon its interaction with antibodies in a micellar medium is dependent on $[H_2O]/[AOT]$ ratio, pH and molarity of polar nucleus, as well as on the initial concentration of antibody. Enzyme peroxidase has been studied in the reverse micellar system of AOT in heptane and in mixed micelle of AOT and Triton X-45 in heptane.[135]

I.6.7 Solar Energy Conversion in Reverse Micelles

For efficient solar energy conversion and storage the separation of photoproducts formed in photosensitized electron transfer reactions is essential. Effective separation of photoproducts can be achieved in a reverse micellar system by vectorial photosensitized electron transfer from a donor in the organic phase to an acceptor in the water pool and vice versa. Laane et al. have demonstrated that a micellar reaction medium promoted the water biophotolysis catalyzed by hydrogenase. The enzyme entrapped in reverse micelle system was shown to generate hydrogen by Ru^{2+} photosensitized electron transfer from the thiophenol donor in the organic solvent, via the methyl viologen (MeV) relay. The microenvironment of reverse micelle stabilizes hydrogenase against inactivation and allows an efficient

photosensitized electron and proton flow from the organic phase to hydrogenase in the aqueous phase.[136]

I.6.8 Cryoenzymology in Reverse Micelles

Reverse micelles also provide convenient media for studying enzyme mediated processes at subzero temperatures. This in turn considerably slows down reactions and thus allows the leisurely examination of the kinetics of formation and decomposition of short lived intermediates. The water-in-oil emulsions stabilize supercooled water droplets against freezing due to heterogeneous nucleation. Cytochrome P-450 and trypsin were shown to retain their activities in surfactant entrapped supercooled water in organic solvents.[137,138]

I.6.9 Nucleic Acids, Plasmids, Bacteria and Mitochondria in Reverse Micelles

Nucleic acids, DNA, RNA, t-RNA can be solubilized without denaturation in hydrocarbon with the help of reverse micelles. High molecular wight DNA (MW = 250,000 Dalton) contained in the reverse micelles can be visualized as suitable model for a head virus. DNA containing reverse micelles offer the possibility of models for condensed packaging of DNA <u>in vitro</u>. Spectroscopic studies on nucleic acids in reverse micelles show no significant changes in the case of low molecular weight samples. With big DNA (20 K Da or above) the change in absorption at 260 nm was explained in terms of the conformational rigidity of the chain.

Plasmids[139] have also been solubilized in reverse micelles. The structural information on plasmid containing reverse micelle is still lacking. Studies of the condon-anticodon interactions in reverse micelles is in progress[140] to assess whether binding in reverse micelles is stronger than in bulk water.

A striking feature of reverse micellar solution is the solubilization of entire cells in it. A reverse micellar system containing polyoxyethylene sorbitan trioleate (TWEEN 85) and water in isopropyl palmitate could solubilize whole cells of E. Coli. The cells remained viable for atleast one day and retained enzyme activity for an even large period of time. There is a possibility of utilizing certain bacterial strains which are able to metabolize hydrocarbons and would utilize the solvent as a carbon source.

Solubilization of mitochondria is very important because these particles, being the chemical factories of the cells, are very interesting from the biotechnological point of view.[141]

I.6.10 Reverse Micelle for Purification of Enzymes from Cells[142]

One of the challenging opening in reverse micellar field is the purification of intracellular enzyme. In the common procedure after cell breakage, the removal of cell debris and nucleic acids are done by methods such as centrifugation, filtration and precipitation. These steps are rather time-consuming. These pre-chromatographic procedures can be replaced by two relatively simple steps. In the first step, bacterial

cells are disintegrated by the surfactant in the reverse micellar medium and in the second step the liberated enzymes are extracted from the reverse micellar phase into an aqueous phase.

I.7 Objective of the Present Study

Our aim to study catalytic efficiency of enzymes in reverse micelle was to find out suitable apolar media for these enzymes and to investigate their kinetic characteristics and properties in reverse micellar solution.

In our studies we have chosen two important enzymes viz. glucose-6-phosphate dehydrogenase and alcohol dehydrogenase. Dehydrogenases are an important class of enzymes that are involved in biological oxidation and reduction and play a significant role in various metabolic mechanism in the living cells such as glycolysis, citric acid cycle, vitamin and co-enzyme metabolism, lipid metabolism, urea cycle etc. Reverse micellar study of these enzymes are important because this versatile medium is able to mimic properties of the cellular environment *in vivo* and has a potential to extend the utility of enzymes in enzyme-mediated organic synthesis.

So far most enzymes of low molecular weight have been solubilized and characterised in reverse micellar solution. Much attention has not been paid to enzymes having high molecular weight (hundred thousand dalton) and made up of two or more sub-units. We have taken big multiple sub-unit enzyme and solubilized them in reverse micellar medium and investigated their properties.

Our studies have given some interesting results. We have successfully solubilized glucose-6-phosphate dehydrogenase in reversed micellar solution of mixed surfactant. On the other hand yeast alcohol dehydrogenase has been solubilised in cationic and anionic reverse micellar solution. Glucose-6-phosphate dehydrogenase was found to be superactive whereas yeast alcohol dehydrogenase showed super activity only in anionic micelles. In all the cases the enzymes obey Michaelis-Menten Equation. Absorption studies established the formation of same product both in aqueous and reverse micellar mdeium. These studies demonstrate that reverse micellar media can be used as an alternative to the aqueous medium for *in vitro* studies of these dehydrogenases which may help to extend the utility of enzymes in general in many fields of science.

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CHAPTER II

PROPERTIES AND CHARACTERISTICS OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE IN REVERSE MICELLES OF MIXED SURFACTANTS

INTRODUCTION

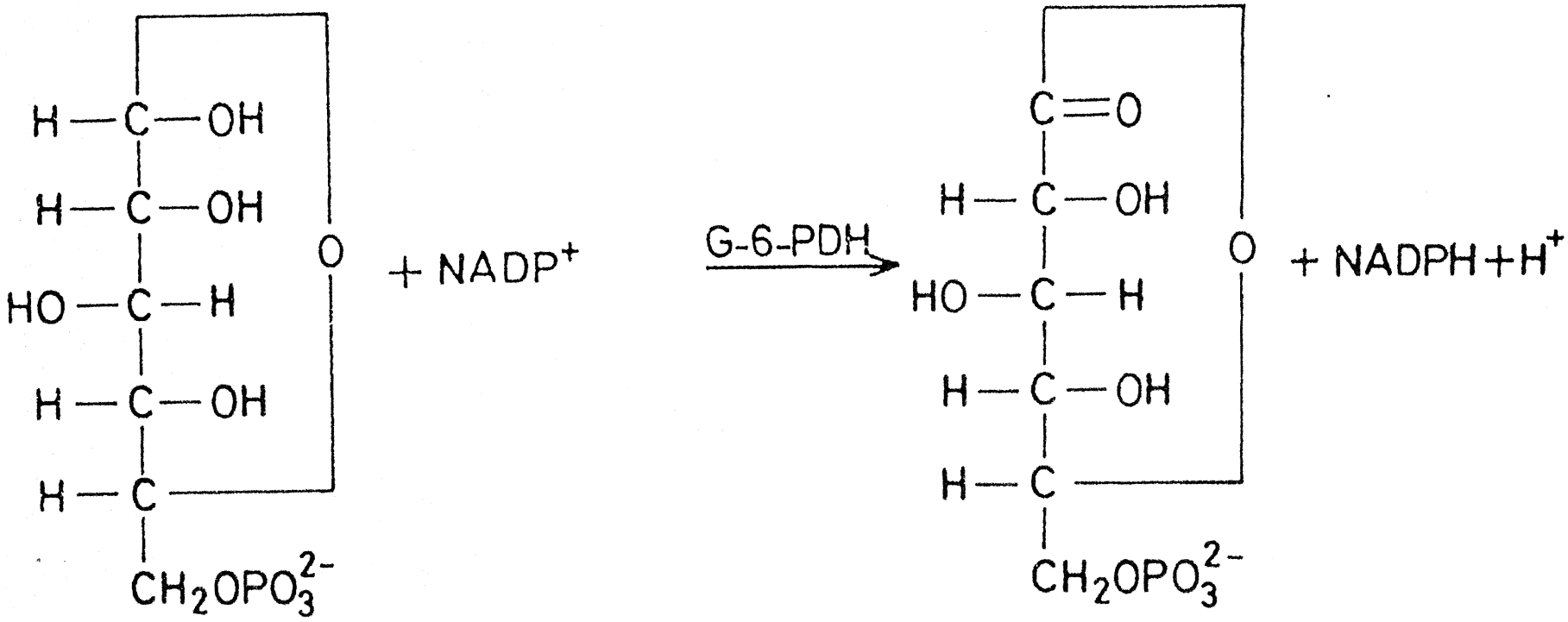
Enzymes are essential for cells in living organism. In cells hundreds of stepwise reactions are controlled by enzymes with high catalytic power and specificity. Enzymes are being increasingly used in chemical industry, in food processing and in agriculture. Several inborn errors of metabolism are known to occur through the lack of (or occasionally, the excess of) certain enzymes. The study of enzymes is very important to comprehend structure and function of enzymes in cell.

For our investigations on behavior of enzymes in reverse micelles in non-aqueous solvents we have chosen an oxidoreductase viz. glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: $NADP^+$ oxidoreductase, EC 1.1.1.49, G-6-PDH). G-6-PDH has been selected primarily because of its varied characteristics such as being made up of multi sub-units, usage in coupled reactions and having very specific involvement in biological processes.

Since it is found that enzymes show different activity and specificity when entrapped in reverse micelles, the optimum conditions for maximum activity in reverse micelles are quite different for different enzymes. In general enzyme investigations in reverse micellar medium are expected to provide a realistic picture of enzyme behavior as the hydrophobic and polar environment created by reverse micelles is more closer to the cellular environment.

Glucose-6-phosphate dehydrogenase (G-6-PDH) is a relatively big enzyme. It is composed of four sub-units in presence of $NADP^+$ and having molecular weight $212x10^3$ dalton. It catalyzes the conversion of glucose-6-phosphate to 6-phosphoglucono-δ-lactone. It is the key enzyme in the pentose phosphate cycle. G-6-PDH is used for the enzymatic determination of $NADP^+$, glucose-6-phosphate, glucose-1-phosphate and for the determination of the activity of phosphoglucose isomerase (PGI), phosphoglucomutase and hexokinase. The mixture with hexokinase is used for the enzymatic determination of glucose. The cause for drug-induced hemolytic anemia has been found to be deficient in glucose-6-phosphate dehydrogenase in red cells. Deficiency in glucose-6-phosphate dehydrogenase is not a rare disease. It is inherited as sex-linked trait.

Glucose-6-phosphate dehydrogenase from yeast has been studied in the reverse micellar solution of sodium bis(2-ethylhexylsulfosuccinate) (AOT) and polyoxyethylene(5) octylphenol (Triton X-45) (1:1 molar ratio) in n-heptane. As this enzyme found to be inactive in cationic (cetyltrimethylammonium bromide,


G-6-PDH
+ NADP+
+ NADPH+H+
Glucose-6-phosphate
6-phosphoglucono-δ-lactone

Scheme II.1

CTAB), anionic (AOT) or nonionic (Triton X-45) reverse micellar solution separately, we have taken reverse micelle of mixed surfactants. The choice of these surfactants is also based on their special characteristics to provide a bigger water pool for big enzyme molecules.

This chapter presents the investigation on the solubilization and kinetic activity of enzyme glucose-6-phosphate dehydrogenase in the reverse micelles formed by AOT and Triton X-45 mixed surfactant in n-heptane medium.

II.2 EXPERIMENTAL SECTION

II.2.1 Materials

Sources of procurement of various chemicals, biochemicals, enzymes and surfactants used are described below:

II.2.1.1 Enzymes and Substrates

Yeast glucose-6-phosphate dehydrogenase (EC 1.1.1.49), grade II (suspension) was a product by the Boehringer Mannheim GmbH, West Germany. Glucose-6-phosphate and $NADP^+$ were obtained from Sigma Chemical Company, St. Louis, MO U.S.A.

II.2.1.2 Surfactants and Solvents

Bis(2-ethylhexyl) sodium sulfosuccinate (AOT) was obtained from American Cyanamid Company, Wayne, N.J., U.S.A. AOT was purified by the method reported in the literature.[1] The purified AOT was cut into small pieces and was dried over P_2O_5 in an

evacuated desiccator for several hours just prior to use. Polyoxyethylene(5) octylphenol (Triton X-45) was obtained from Rohm & Haas, Philadelphia, U.S.A. n-Heptane, HPLC and spectroscopic grade was the product of s.d.fine.chem pvt. ltd. Boisar, India.

II.2.1.3 Other Chemicals

The buffer component glycine was purchased from Sigma Chemical Co. U.S.A. and potassium hydroxide was from Merck, India.

II.2.2 Preparation of Enzyme and Substrate Reverse Micellar Solutions

Homogeneous (optically transparent, i.e. non turbid) solution of enzyme and substrates in reverse micelles may be obtained by different methods viz. injection method[2], phase transfer method[3] and solid phase extraction method.[4] We have followed the injection method which is the simplest, and the one most commonly used for the preparation of enzyme and substrates containing reverse micelles. According to this method, concentrated stock solutions of enzymes, substrates etc., prepared in aqueous buffer at desired pH were injected with microsyringes (Hamilton Co. USA) into the AOT-Triton X-45 (1:1)/n-heptane solution. The desired water content was set by an additional injection of the buffer solution into the reverse micellar solutions and the resulting mixture was shaken vigorously on a vortex mixer for a few tens of seconds until the formation of a homogeneous (optically transparent) solution.[2,5]

The buffer used to prepare the stock solutions of enzyme, coenzyme and substrate was glycine-potassium hydroxide. pH was measured on ELICO LI-120 digital pH-meter at 30°C. The concentrations of the enzyme, coenzyme, substrate and buffer components were adjusted according to the ease of solubilization at specified water pools.

II.2.3 Measurement of Activity of the Enzyme

Gilford response and Gilford 260 UV/Vis spectrophotometers were used to measure the activity of the enzyme in reverse micellar solution. The temperature of the cell compartment was kept at 30 ± 0.1°C by circulating water in the thermospacer set of the spectrophotometer from an external thermostat which controlled temperature by a high precision electronic relay. The reverse micellar solutions containing substrate, coenzyme and desired amount of buffer, were incubated for few minutes and the reaction was started by injecting 2-8 μl of aqueous stock solution of enzyme to 1.0 ml of the incubation mixture. This micellar solution was taken into a 1.5 ml quartz cell of 1.0 cm path length and the initial velocity of enzyme reaction was measured by observing the increase in absorbance with time at the absorption maximum (340 nm) of NADPH. Adequate controls were run to ascertain that the increase in absorbance was not an artifact due to precipitation or separation of water in reverse micellar solution.

II.2.4 Calculation of Enzyme Activity

One unit of enzyme is defined as the amount of enzyme that will form 1μ mole of NADPH per minute. Specific activity is defined as the number of units per milligram of protein.

By Beer-Lambert's Law

$$\text{Absorbance } (A) = ECl \text{ or } C = \frac{A}{El}$$

where E = extinction coefficient, C = concentration in moles/litre, l = path length in cm.

Here E^{NADPH} at 340 nm = 6.2×10^3 litre $mole^{-1}$ cm^{-1} & l = 1.0 cm.

$$\text{Hence } C = \frac{A_{340\ nm}/min}{6.2 \times 10^3 \times 1} \text{ (mole/litre) } min^{-1}$$

$$C = \frac{A_{340\ nm}/min}{6.2} \text{ (}\mu\text{ mole/ml) } min^{-1}$$

Specific activity = μ mole product formed $min^{-1} mg^{-1}$ protein

$$= \frac{A_{340\ nm}/min}{6.2 \times \text{mg enzyme/ml reaction mixture}}$$

II.3 RESULTS AND DISCUSSION

For <u>in vitro</u> study of enzyme, reverse micellar solution was found to be an useful medium. Buffer added to the reverse micellar solution goes to the inner core of the micelle and thus

forms the water pool. Enzymes are entrapped inside this water pool and protected from the harmful action of organic solvents and surfactant molecules. This is how enzymes are made to work in the microcaptive environment. Here micelles behave as novel microreactors which accommodate enzymes and all the substrates (water soluble) in the water pool.

II.3.1 Solubilization of Glucose-6-phosphate Dehydrogenase in Reverse Micelles

The solubilization of enzyme in reverse micellar solution was found to be dependent on surfactant concentration, the molar ratio of water to surfactant (w_o), pH of buffer and the nature and concentration of its constituents, concentration of aqueous stock solution of enzyme, temperature etc. We have solubilized glucose-6-phosphate dehydrogenase in reverse micellar solution of cationic, anionic, nonionic and mixed surfactants. The reverse micellar solution of AOT and Triton X-45 in n-heptane was found to be quite suitable for solubilization of yeast glucose-6-phosphate dehydrogenase. This water soluble enzyme forms a homogeneous and optically transparent reverse micellar solution of AOT/Triton X-45 (1:1) in n-heptane under specific conditions. This implies that this hydrophilic enzyme is solubilized in reverse micellar solution according to water-shell model wherein the enzyme resides in the centre of the water pool, surrounded by shell of water molecules which protects the enzyme from the surfactant wall and from the bulk organic solvent. It is expected that only a small fraction of the micelles are occupied by enzyme molecules, the rest being available for coenzyme,

substrate and other reactant molecules. These enzyme containing micelles, coenzyme, substrate containing micelles and the micelles containing enzyme-substrate complex, together with the unoccupied micelles are considered to be in rapid equilibrium i.e. exchanging guest molecules very rapidly.

In the reverse micellar solution concentration of solubilized enzyme, substrate or coenzyme can be expressed in two different ways. One is relative to the water pool, where enzyme reaction takes place and the other is relative to the overall system. For a compound which is practically insoluble in the organic solvent, we can consider that the compound is located in the water pool and the two concentrations are related as follows.[6]

$$C_{ov} = C_{wp}F_w$$

where C_{ov} and C_{wp} are the overall concentration and the water pool concentration respectively and F_w is the water volume fraction which is given as

$$F_w = w_o \text{ [Surfactant](1.8)/100}$$

The solubility of glucose-6-phosphate dehydrogenase depends on the ionic strength of the enzyme solution. The stock solution of glucose-6-phosphate dehydrogenase is a suspension in ammonium sulphate solution of 3.2 mol/l at approx. pH = 6. It is a concentrated solution of 5.0 mg/ml. The enzyme was diluted 100 times in 100 mM glycine-KOH, pH=9.4 and then added to the reverse micellar solution. The solution becomes clear by vortexing for

5-10 seconds. The concentration of enzyme (0.1 μ g/ml) was adjusted in the reverse micellar solution such that the change in optical density for the enzymatic reaction was measurable spectrophotometrically. Generally solubility of enzyme in reverse micelle is affected by its size. Yeast glucose-6-phosphate dehydrogenase is a relatively big enzyme of 4 sub-units[7] (M.W.=212 x 10^3 dalton in presence of $NADP^+$). Solubility of this enzyme in the reverse micellar solution of mixed surfactants is dependent on the concentration of buffer and type of the buffer. Here the buffer (100 mM glycine-KOH) which has been used to carry out its assay in aqueous medium was found to be suitable for the assay in reverse micelles too. This is not true for all the enzymes.

II.3.2 Solubilization of Buffer in Reverse Micellar Solution of Mixed Surfactants (AOT and Triton X-45 in n-Heptane)

After achieving solubilization of enzymes in reverse micellar solution it was essential to find out the amount of buffer that goes into this solution. For the present work we have used 0.1 M solution of AOT and 0.1 M Triton X-45 in n-heptane. Fig. II.1 shows the variation of amount of buffer solubilised as a function of pH. Below pH 9.2, buffer solubilized is less than 24% whereas in the range of pH 9.4-9.7, the amount increases to as much as 40%. The solubility of buffer decreases sharply beyond 9.7 and remain unchanged at 20% after pH 10. One of the interesting point is that this reverse micellar solution of AOT and Triton X-45 takes in more amount of buffer compare to that of water. It solubilizes only 17% of

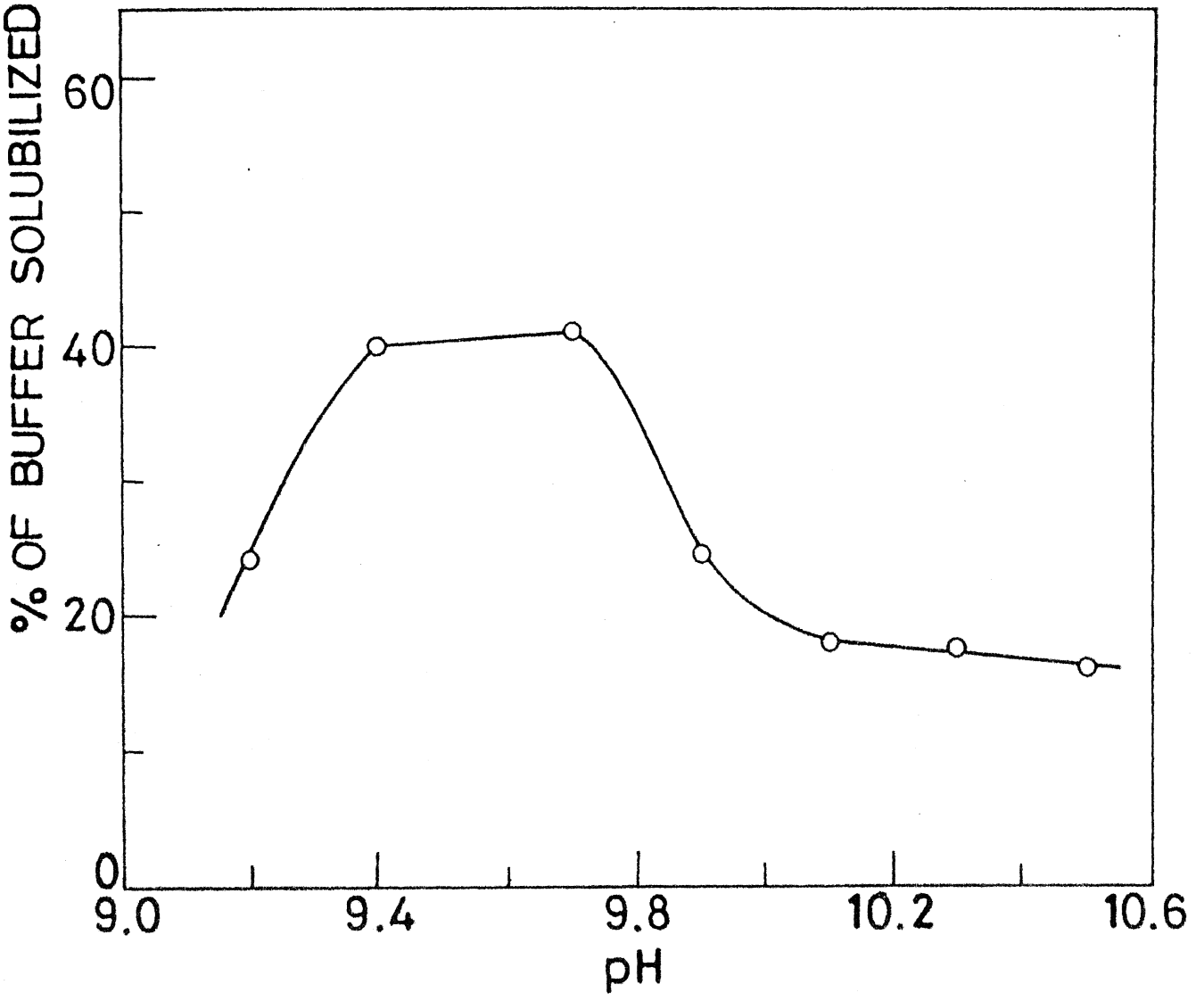


Fig.II.1 Percentage of buffer solubilized in mixed micelles of 0.1M AOT and 0.1M Triton X-45 in n-heptane as a function of pH of the buffer; Buffer was 100 mM glycine-KOH.

water. It is contrary to general observation. Another notable feature is that though the inner core of the reverse micellar solution is negatively charged it prefers to take in the buffer of alkaline pH which contains more negatively charged anions.

II.3.3 Enzyme Activity in Reverse Micellar Solution

Glucose-6-phosphate dehydrogenase from yeast was found to maintain its catalytic activity in reverse micellar solution of mixed surfactant (AOT and Triton X-45) in n-heptane. This reverse micellar solution protects this enzyme from the harmful effect of the organic solvent. The activity of this enzyme is very much dependent on the degree of hydration of this surfactant. Degree of hydration is also expressed as water pool (w_o) which is defined as the ratio of molar concentration of water to surfactant concentration. To optimize the conditions for maximum activity in reverse micellar solution, the effect of degree of hydration, pH, surfactant concentrations etc. were studied systematically.

II.3.3.1 Effect of Degree of Hydration (w_o) on Enzyme Activity

Fig. II.2 shows the variation of specific activity of the enzyme with the change of degree of hydration. The maximum activity of this enzyme in aqueous buffer was found at pH = 9.4. The enzyme was found to be completely denatured when w_o is less than 25. As degree of hydration increases beyond 25, the enzyme starts to manifest its activity in reverse micellar solution. When degree of hydration is more than 30.6, this enzyme is superactive in this reverse micellar solution. This is one of

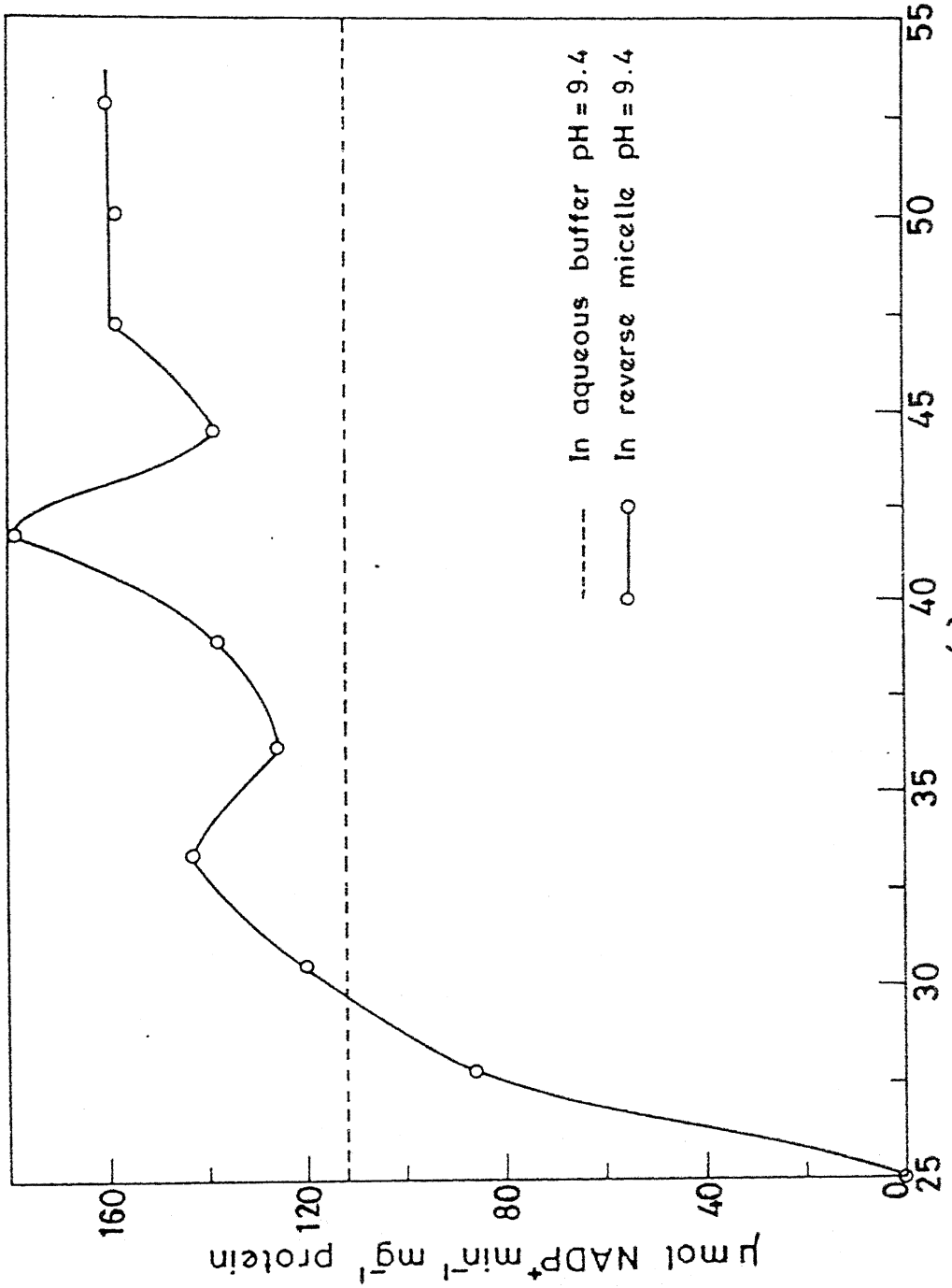


Fig.II.2 Specific activity of G-6-PDH in mixed micelles of AOT and Triton X-45 in n-heptane as a function of w_o. The concentrations were [AOT] = 0.1M; [Triton X-45] = 0.1M; $[NADP^+]$ = 0.11 mM; [G-6-P] = 0.35 mM. Buffer was 100 mM glycine-KOH; pH = 9.4. [G-6-PDH] = 0.1 μg/ml.

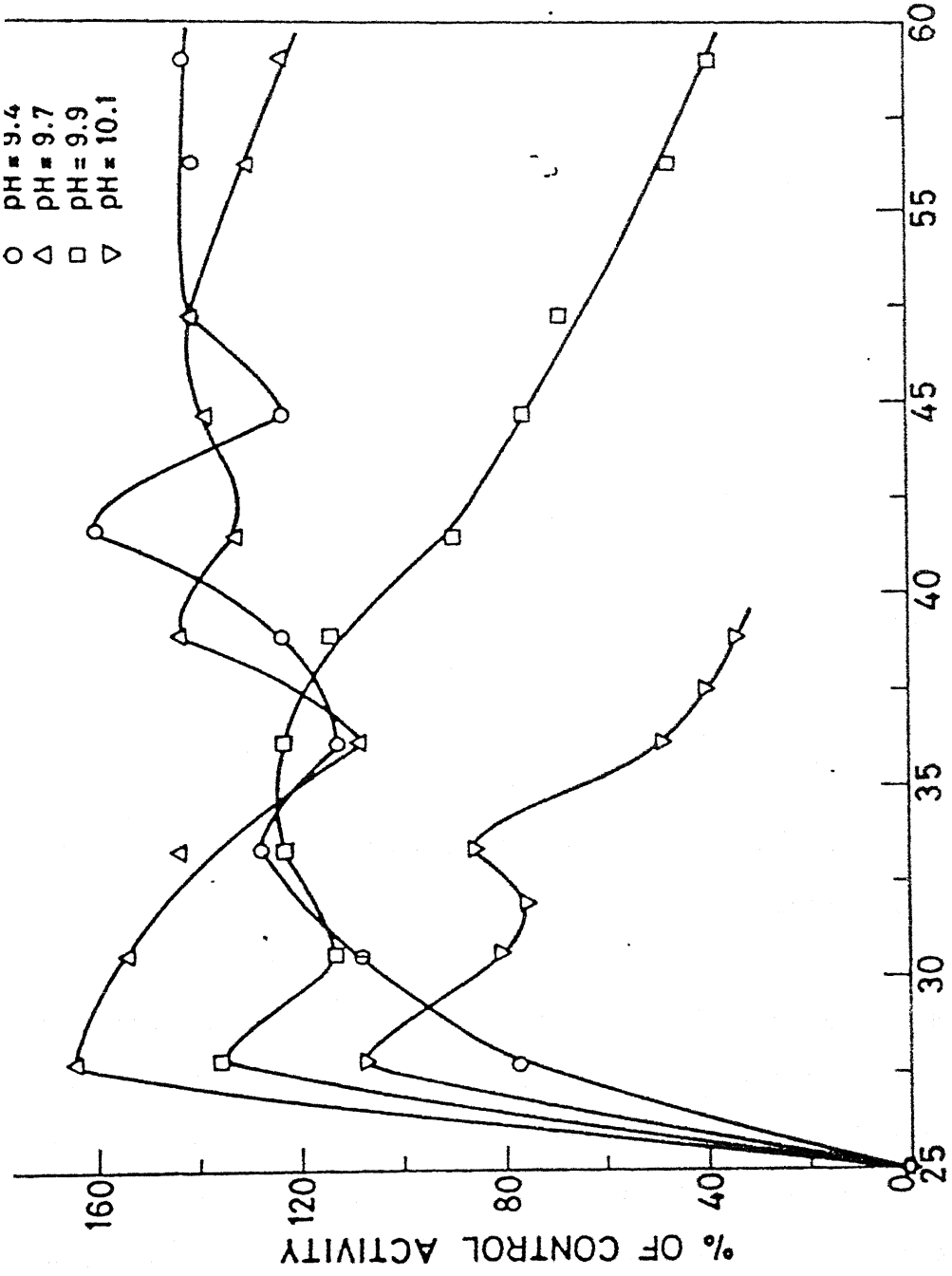


Fig.II.3 Activity of G-6-PDH in mixed micelles of AOT-Triton-45-n-heptane versus degree of hydration (w_o). The activity is expressed relative to the activity in aqueous buffer. Other concentrations were same as in Fig. II.2.

the rare phenomenon in reverse micellar enzymology. Super activity means that enzyme activity is higher than that in aqueous buffer medium at its optimum conditions. This enzyme maintains its super activity at all the water pools beyond 30.6. The activity versus degree of hydration (w_o) of the surfactant profile exhibits bell shaped curves centred on two optimal degree of hydration. Here the first optimal degree of hydration ($w_{o,opt}^{first}$) is at the value of w_o = 33.3 and activity 127.69% and the second optimal degree of hydration ($w_{o,opt}^{second}$) is at the value of w_o = 41.7 and activity 160%.

Fig. II.3 is the plot of percent control activity of glucose-6-phosphate dehydrogenase as a function of degree of hydration at different pH. Here pH, means pH of the stock buffer solution. At all pH values, the plot was found to be bell-shaped curve having two maxima. As the water pool size increases the activity of the enzyme also increases and after showing two maxima, the activity at higher w_o flattens out or decreases. At pH 9.4 to 9.7 where the reverse micellar solution can solubilize maximum amount of water, the enzyme shows super activity when w_o is more than 30.6. This enzyme shows maximum activity (164% of control) at w_o = 27.8, pH 9.7. In the present study the term pH has been used in place of pH stock. This pH stock may or may not be the actual pH inside the core of reverse micelles. The plot of activity versus w_o has been found to be bell-shaped, for many many enzymes viz. α-chymotrypsin,[6] alkaline phosphatase,[8] lipase,[9] lysozyme,[10] glutathione reductase,[11] lactate dehydrogenase,[12] malate dehydrogenase[5] etc. The bell-shaped dependence of enzyme activity upon w_o represents a general

feature of the enzyme behaviour in micellar solution. In the present case too we get bell-shaped curve but it is centred on several optimal degrees of hydration. This is an unusual observation. So far there is only one report[13] of swine muscle lactate dehydrogenase (LDH) in AOT/octane, where bell-shaped curves centred on several optimal degrees of hydration were observed. These optima are attributed to the entrapment of dimers, tetramers and octamers (i.e. 2-tetramers) of LDH in the reverse micelles as a function of micelle size which in turn is a function of hydration. The same interpretation could be given in the case of yeast glucose-6-phosphate dehydrogenase which is an oligomeric enzyme of four sub-units in the presence of $NADP^+$.

II.3.3.2 Effect of pH on Enzyme Activity

The rate of an enzymatic reaction and its characteristic kinetic parameters depend on the pH of the buffer solution. When an enzyme is transferred from aqueous buffer solution to the water pool of the reverse micellar solution, the obvious expectation is that the reaction will depend on the pH of the water pool. Fig. II.4 shows the variation of specific activity of G-6-PDH with change of pH in aqueous buffer and in reverse micelle. In this study the pH in reverse micellar solution is the pH of the stock (pH_{stock}) buffer solution which is transferred in reverse micelle to create water pool. In aqueous buffer this enzyme shows fairly good activity in the range 8.6-9.9 and maximum at pH = 9.4. In reverse micelle the enzyme does not show any activity below 9.2. As pH increases from 9.2 there is sharp increase of activity and at pH = 9.7 it shows maximum activity.

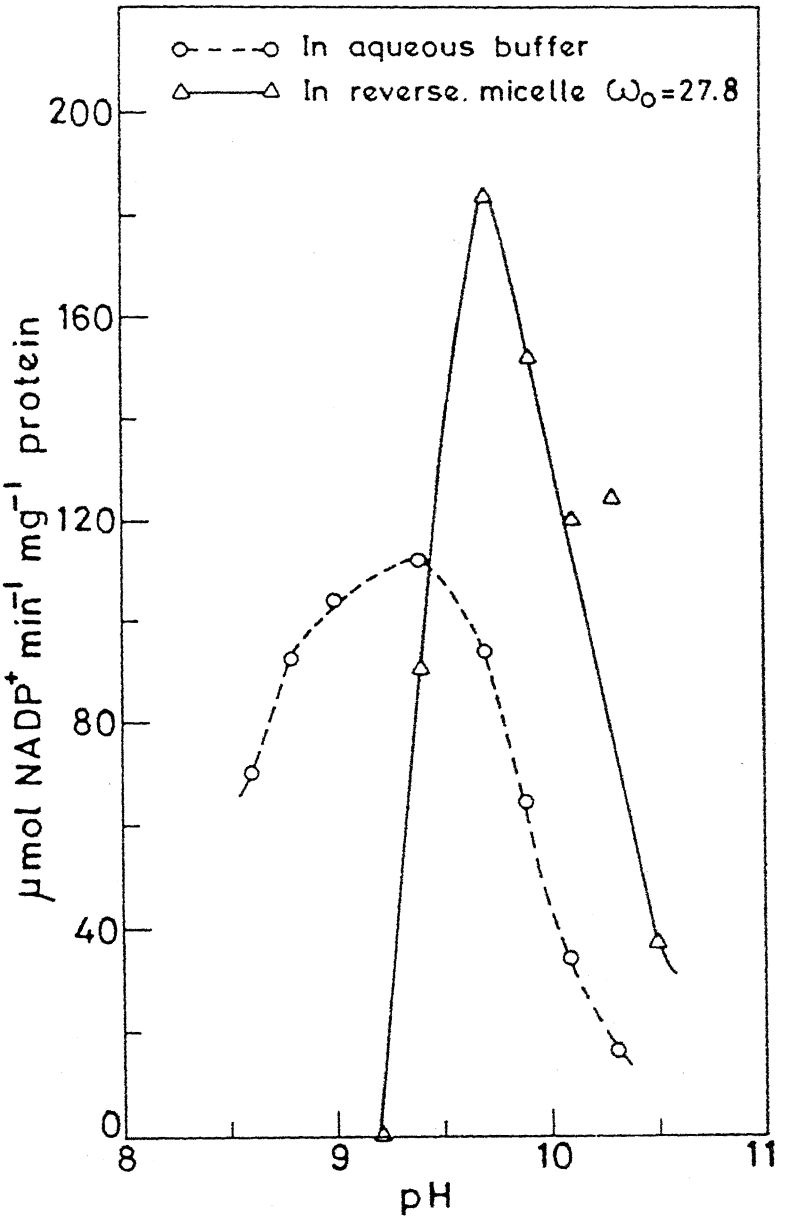


Fig.II.4 Dependence of G-6-PDH activity on the pH of the aqueous solution (o—o) and of the buffer transferred to the micellar solution of mixed surfactants of AOT and Triton X-45 in n-heptane (—). The concentrations were [AOT] = 0.1M; [Triton X-45] = 0.1M; [$NADP^+$] = 0.11 mM; [G-6-P] = 0.35 mM. Buffer was 100 mM glycine-KOH; [G-6-PDH] = 0.1 μg/ml.

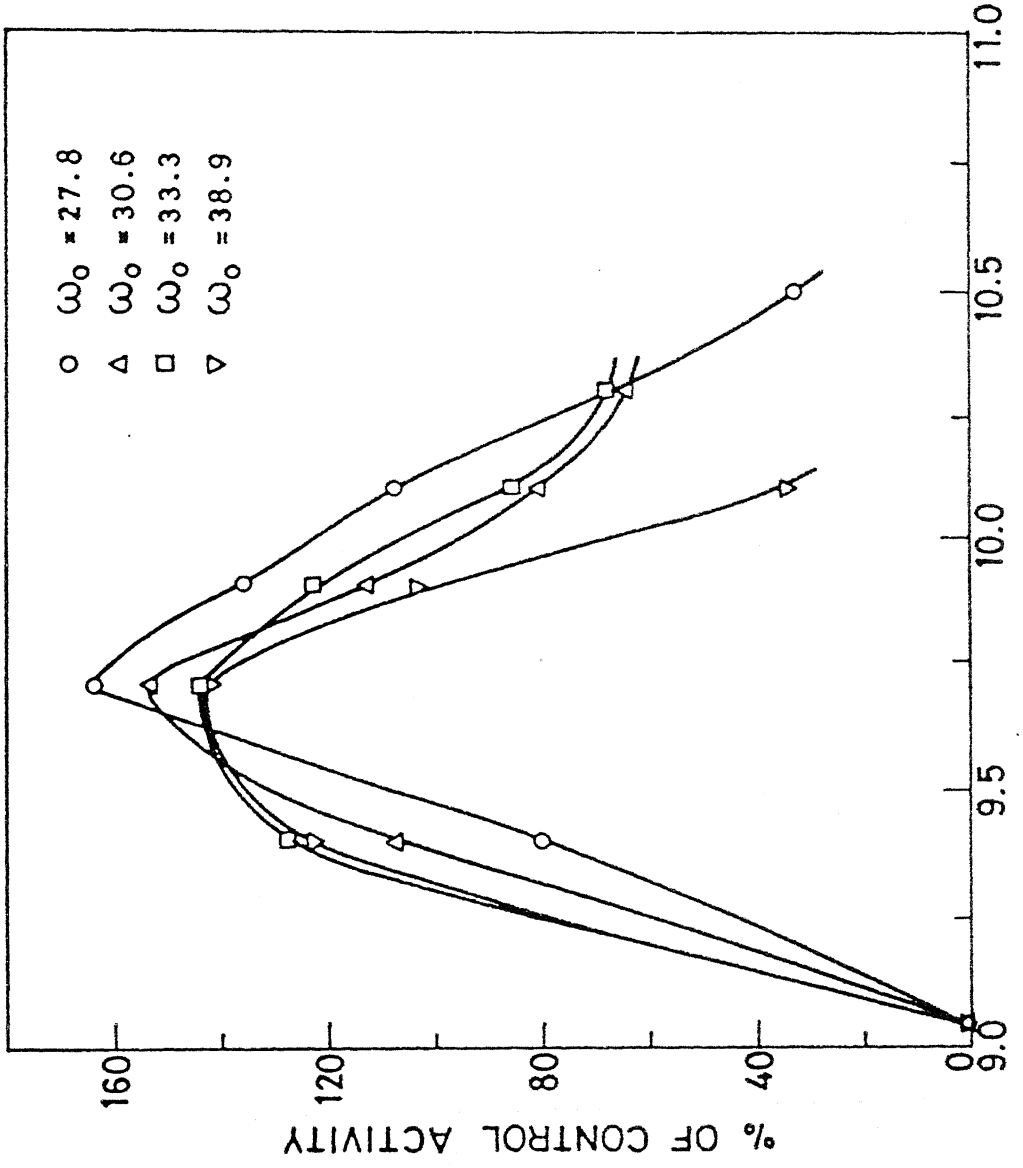


Fig.II.5 Activity profile of G-6-PDH in AOT-Triton X-45-n-heptane reverse micelle as a function of pH at different water pools. The activity is expressed relative to the activity in aqueous buffer (100 mM glycine-KOH, pH = 9.4). Other concentrations were same as in Fig. II.4.

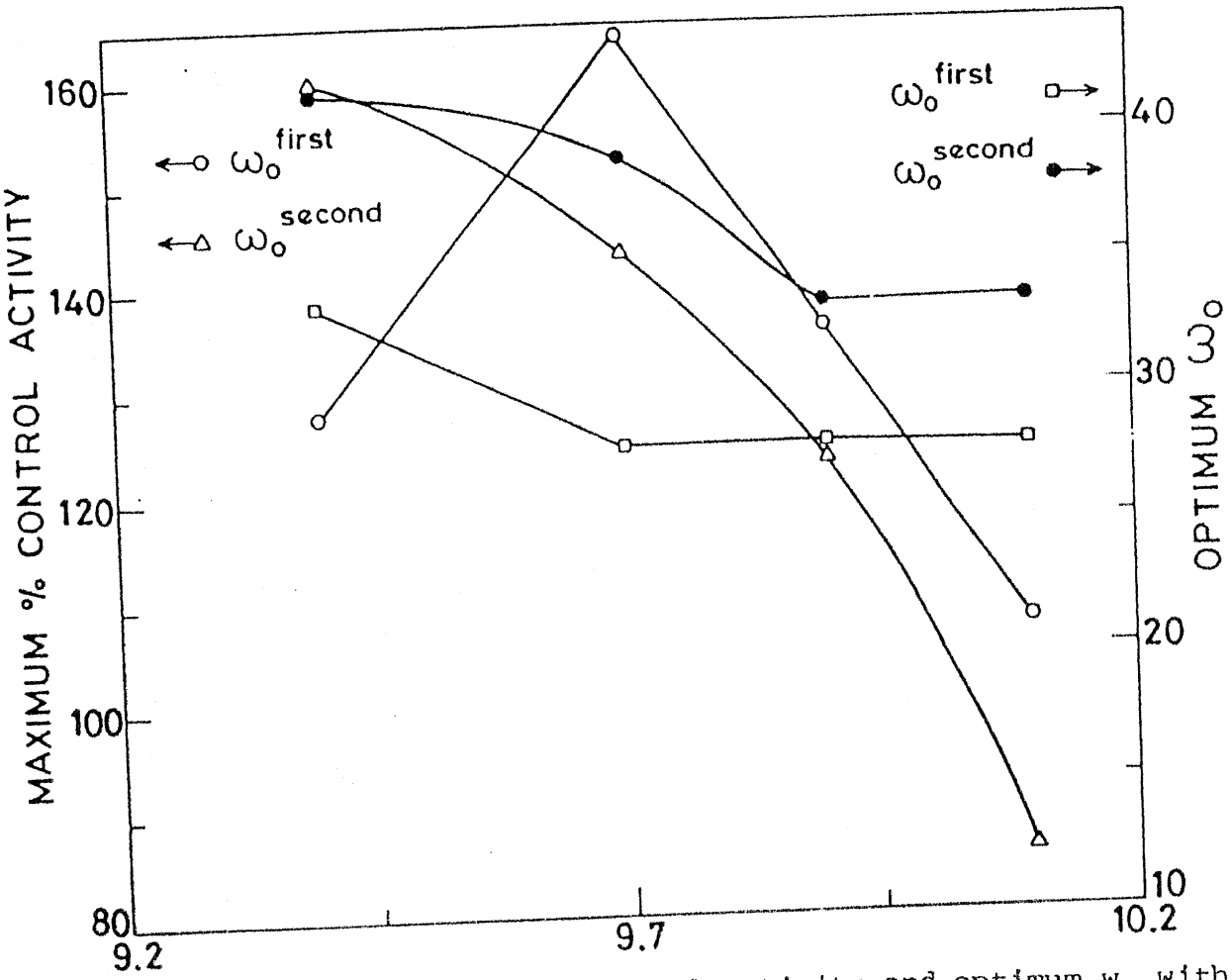


Fig.II.6 Variation of maximum percentage control activity and optimum w_o with the pH for G-6-PDH in AOT-Triton X-45 reverse micellar system. The concentrations for different components were same as in Fig. II.4.

A further increase of pH results in a sharp decline in the activity. The pH for maximum activity is shifted slightly to higher value compared to aqueous buffer. In general it is found that pH profile for enzymes in reverse micelles shifts to the alkaline side.[5,6,10]

The variation of activity of G-6-PDH with change of pH at fixed values of w_o is shown in Fig. II.5. The plots at different water pools are bell-shaped. At all the water pools the maximum activity is observed at pH = 9.7. In the pH range 9.5-9.9, this enzyme shows super activity at different water pools. At pH below 9.0, the enzyme does not show any activity. The maximum activity obtained is 164% at pH = 9.7 and water pool 27.8.

An interesting feature of G-6-PDH is that it shows more than one optimum w_o which are designated as $w_{o,opt}^{first}$ (first w_o at which it shows optimum activity), $w_{o,opt}^{second}$ (second w_o at which it shows optimum activity) etc. Variation of maximum % control activity and optimum w_o ($w_{o,opt}^{first}$ and $w_{o,opt}^{second}$) with pH are plotted in Fig. II.6. Maximum % control activity increases with increase of pH for $w_{o,opt}^{first}$, reaches a maximum and then decreases. Whereas in case of $w_{o,opt}^{second}$ maximum control activity sharply decreases with increase of pH. With increase of pH, both $w_{o,opt}^{first}$ and $w_{o,opt}^{second}$ slowly decrease and flatten out to a constant value of w_o.

II.3.3.3 Effect of Surfactant Concentration on Eyzyme Activity

Surfactant concentration is one of the critical parameters in the determination of the optimum conditions for enzyme reaction in reverse micellar solution. In the present study we

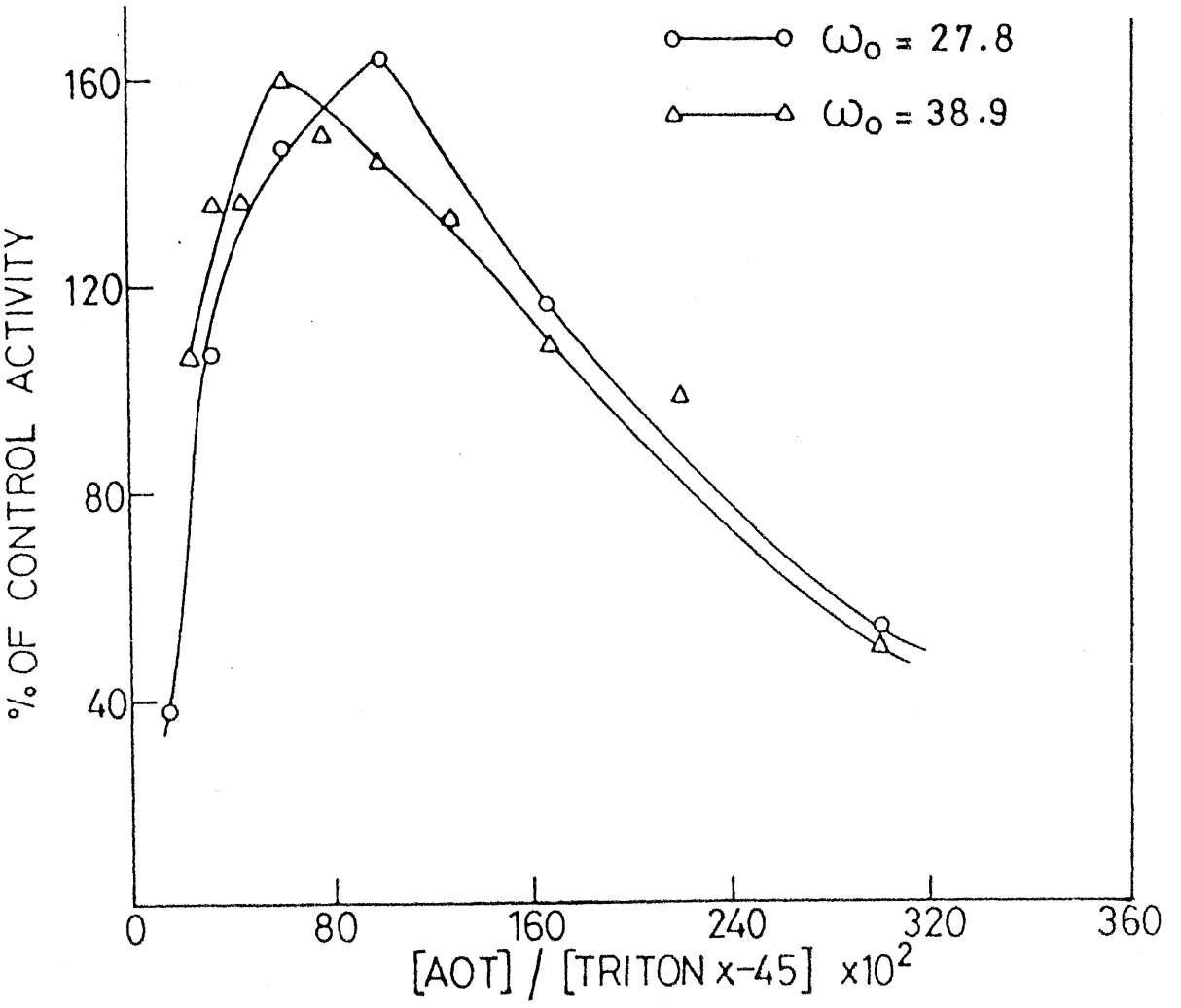


Fig.II.7 Dependence of G-6-PDH activity on ratio ([AOT]/[Triton X-45]) of surfactant concentration at w_o = 27.8, pH = 9.7 and w_o = 38.9, pH = 9.7 in AOT-Triton X-45-n-heptane reverse micellar system. The concentrations were [G-6-P] = 0.35 mM, [$NADP^+$] = 0.11 mM and [G-6-PDH] = 0.1 µg/ml. Buffer was 100 mM glycine-KOH.

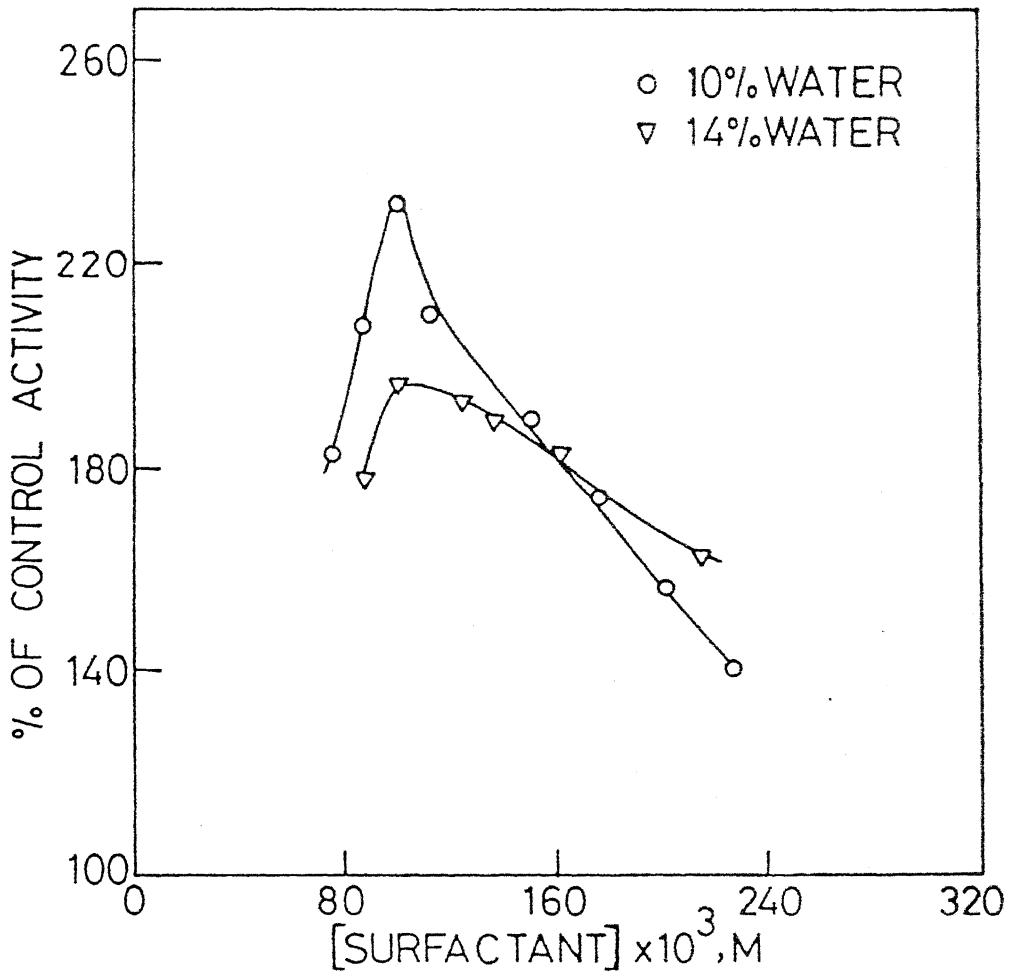


Fig.II.8 Effect of total surfactant concentration ([AOT] + [Triton X-45]) on G-6-PDH activity in AOT-Triton X-45-n-heptane reverse micellar system at 10% water, pH = 9.7, [AOT]/[Triton X-45] = 1 and at 14% water, pH = 9.7, [AOT]/[Triton X-45] = 1. Other concentrations were same as in Fig. II.7.

have used reverse micellar solution of mixed surfactant. The effect of surfactant concentration can be studied in three different ways viz. (a) Changing the ratio of surfactant concentration keeping water pool size (w_o) and total surfactant concentration (0.2 M) constant. (b) Varying the total concentration of surfactant ([AOT] + [Triton X-45]) when ratio of two surfactant concentration is constant. (c) Changing the total surfactant concentration and keeping ratio of surfactant concentration and water pool size invariant.

(a) Enzyme activity is plotted as a function of the ratio of concentration of AOT and Triton X-45 in Fig. II.7 at water pools 27.8 and 38.9. The figure shows that the enzyme is most active when concentration of AOT and Triton X-45 are nearly equal. In this ratio enzyme is superactive. On the other hand at high concentration of AOT or at high concentration of Triton X-45 the enzyme shows poor activity.

(b) The second variation of surfactant concentration effect was carried out by changing total concentration of the surfactants when their ratio is one. The investigations were done at 10% water and 14% water. Though the percentage of water is kept constant change of total surfactant concentration changes water pool size of the system. Fig. II.8 is the plot of % of control activity vs total surfactant concentration and it reveals a striking feature that as long as ratio of surfactant concentration is one, the enzyme is superactive. Another important observation is that with increase of total surfactant concentration enzyme activity increases, reaches maximum and

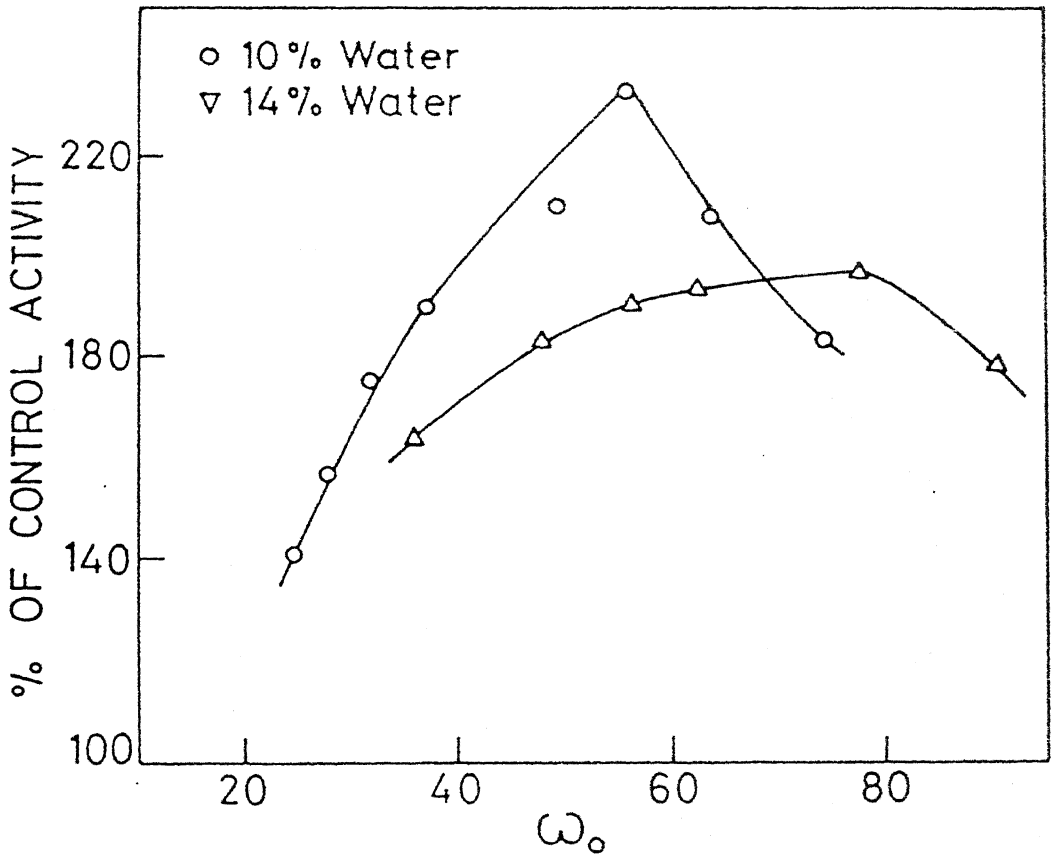


Fig.II.9 Effect of water pool (w_o) on G-6-PDH activity in AOT-Triton X-45-n-heptane reverse micellar system at 10% water, pH = 9.7, [AOT]/[Triton X-45] = 1 and at 14% water, pH = 9.7, [AOT]/[Triton X-45]=1. Other concentrations were same as in Fig. II.7. The water pool change were effected changing total surfactant concentration as in Fig. II.8.

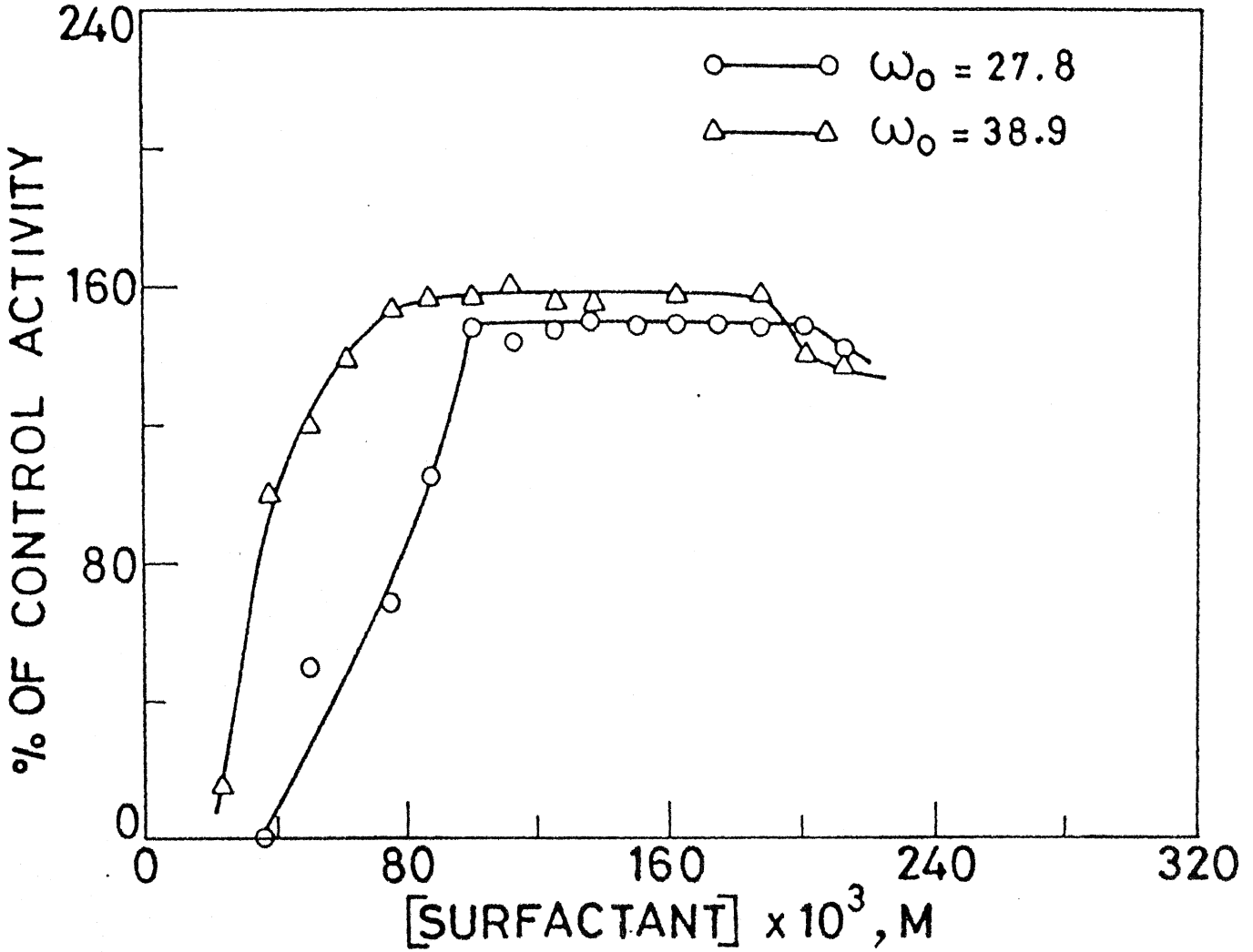


Fig.II.10 Dependence of G-6-PDH activity on total surfactant concentrations in AOT-Triton X-45-n-heptane reverse micellar system at w_o = 27.8, pH = 9.7, [AOT]/[Triton X-45] = 1 and w_o = 38.9, pH = 9.7, [AOT]/[Triton X-45] = 1. Other concentrations were same as in Fig.II.7.

again falls off with further increase of total surfactant concentration. Thus the effect of variation of total surfactant concentration is similar in nature to that of a single surfactant reverse micellar solution. The plots are bell-shaped and show maximum activity in the vicinity of 0.1 M of total surfactant concentration. The maximum activity obtained is 233% at 0.1 M of total surfactant concentration where w_o = 55.6.

Fig. II.9 shows % of control activity as a function of water pool size when total surfactant concentration is changed as in Fig. II.8. It indicates that the enzyme can work in the water pool range larger than 25 and at all water pools the enzyme is superactive as long as the concentration of the two surfactants is same. Moreover, like single surfactant micellar solution the plot is bell-shaped and the activity is less at low water pool size and higher water pool size but in the intermediate range the activity attains a maximum value.

(c) Fig. II.10 is the plot of percentage control activity against total surfactant concentration when ratio of two surfactant concentration and water pool size are invariant. The result indicates that at lower concentration of total surfactant concentration the activity is less. With increase of total surfactant concentration activity increases and then attains a saturation value. In the range of 0.1M-0.18M total surfactant concentration the activity of enzyme reaches its maximum value for the particular water pool size.

II.3.3.4 Spectral Study of Glucose-6-phosphate Dehydrogenase Reactions

Spectral study is a powerful tool for obtaining structural information. UV/Vis spectroscopy has been used most extensively for the biochemical studies.[14] NADPH and $NADP^+$ (nicotinamide-adenine dinucleotide phosphate and its reduced form) are the two coenzymes which are involved in oxidation-reduction reaction catalyzed by glucose-6-phosphate dehydrogenase. The ultraviolet absorption spectrum of the oxidised form show only a band at 260 nm due to purine and pyridine rings, but the reduced form show an additional band at 340 nm. It is widely accepted that spectral properties of a compound are significantly affected by the environment. Guest molecules in reverse micellar environment are therefore, expected to show significant changes in their spectral characteristics. G-6-PDH, G-6-P and $NADP^+$ form a clear solution in reverse micelles of AOT-Triton X-45/n-heptane. Hence absorption spectroscopy is found to be a convenient tool for studying the enzyme reactions in microheterogeneous medium.

To demonstrate the formation of identical product in aqueous (buffer medium) and reverse micellar medium for G-6-PDH catalyzed reaction, absorption spectra of $NADP^+$, NADPH and product formed, in both the media were recorded (Fig. II.11, Fig. II.12 & Fig. II.13). In aqueous solution at pH = 9.4 the absorption maxima of $NADP^+$, NADPH and product are at 260 nm, 260 and 340 nm, 260 and 340 nm respectively whereas in the reverse micellar medium these are at 340 nm, 340 nm respectively. There is no peak at 260 nm for the coenzymes in this reverse micellar system. One peak of

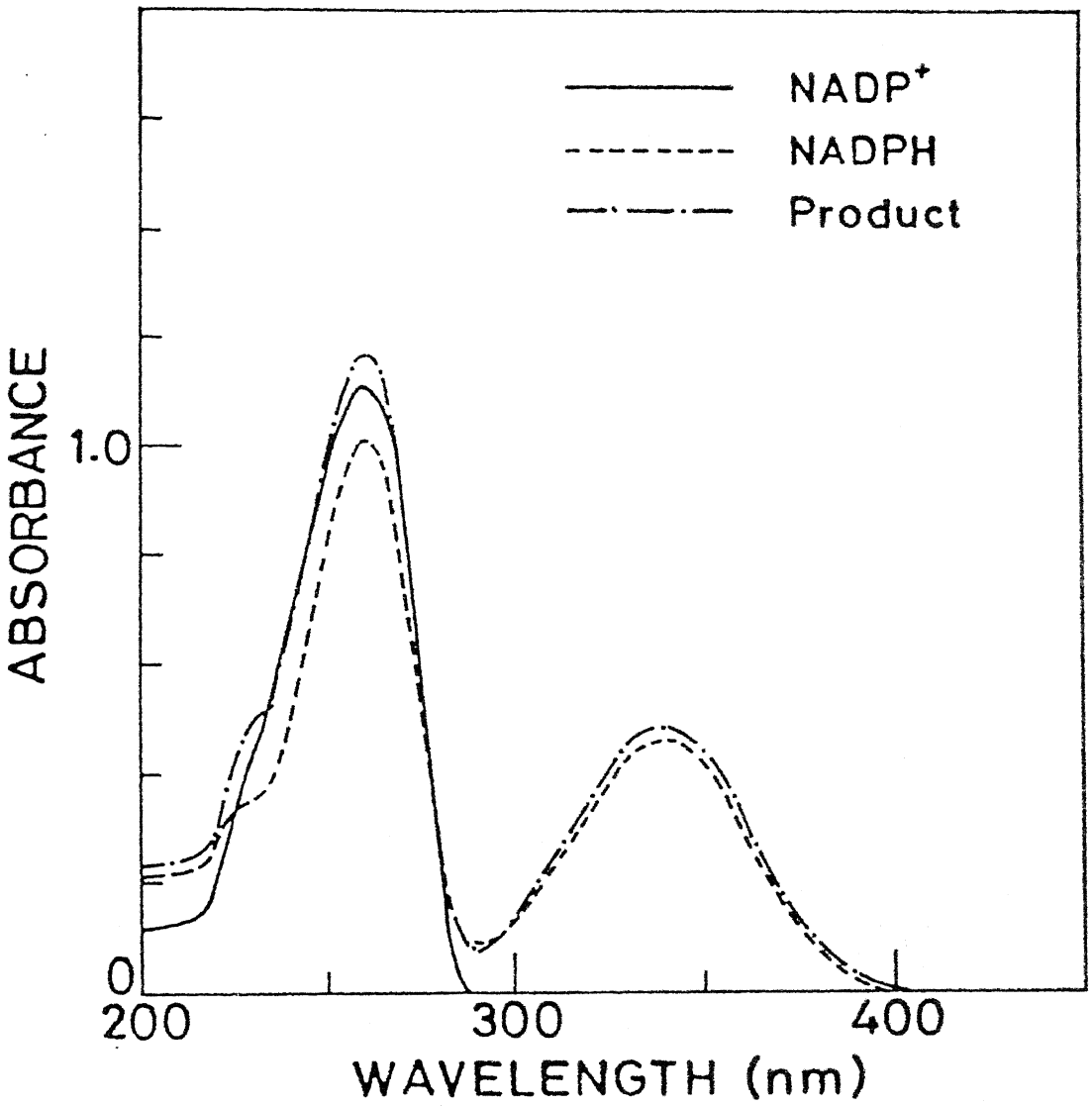


Fig.II.11 Electronic absorption spectra of $NADP^+$, NADPH and the product formed (after the completion of G-6-PDH catalysed reaction) in aqueous buffer (100 mM glycine-KOH, pH = 9.4).

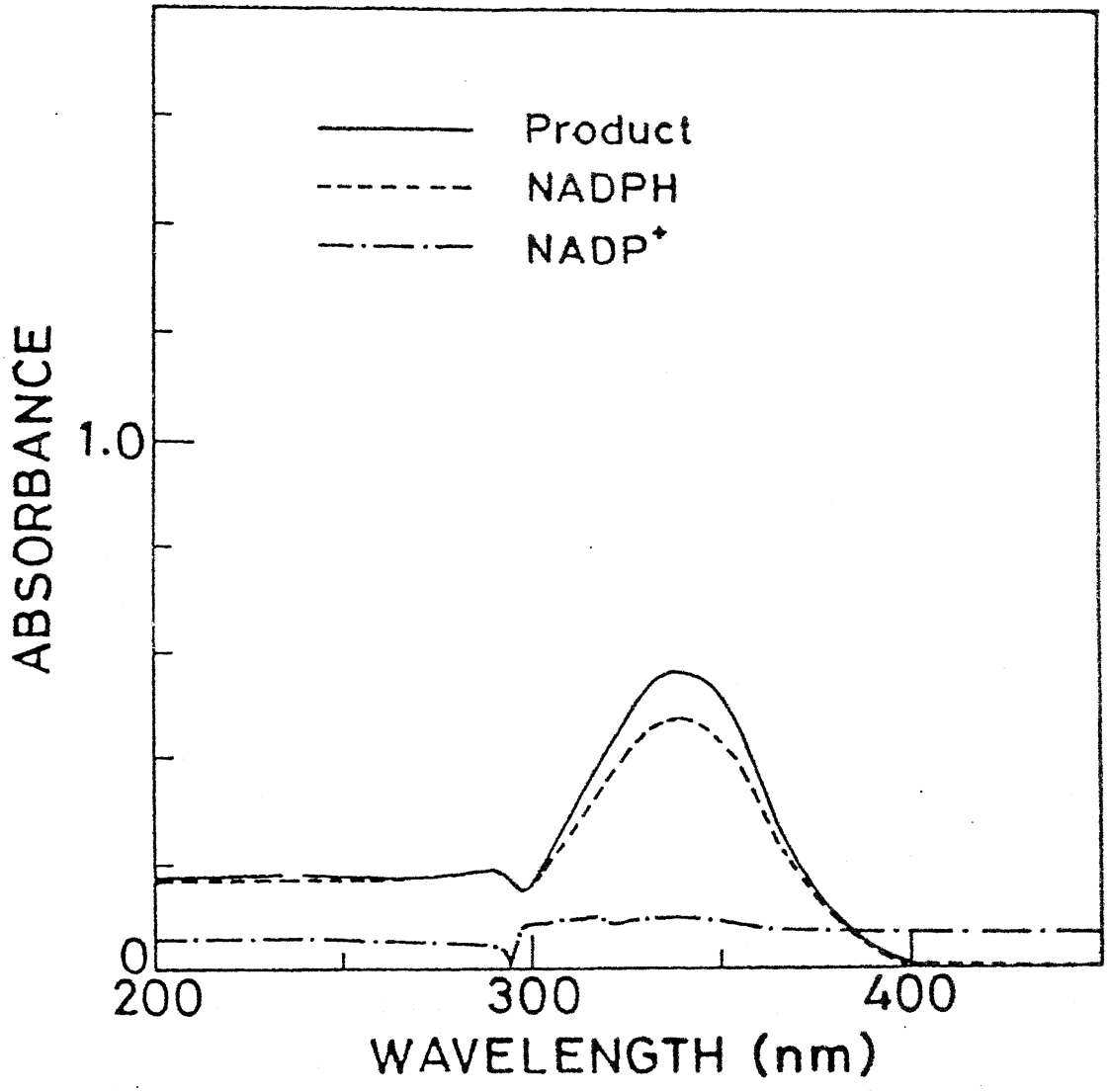


Fig.II.12 Electronic absorption spectra of $NADP^+$, NADPH and the product formed (after the completion of G-6-PDH catalyse reaction) in micellar solution of AOT-Triton X-45-n-heptane at w_o = 27.8, pH = 9.7 (100 mM glycine-KOH).

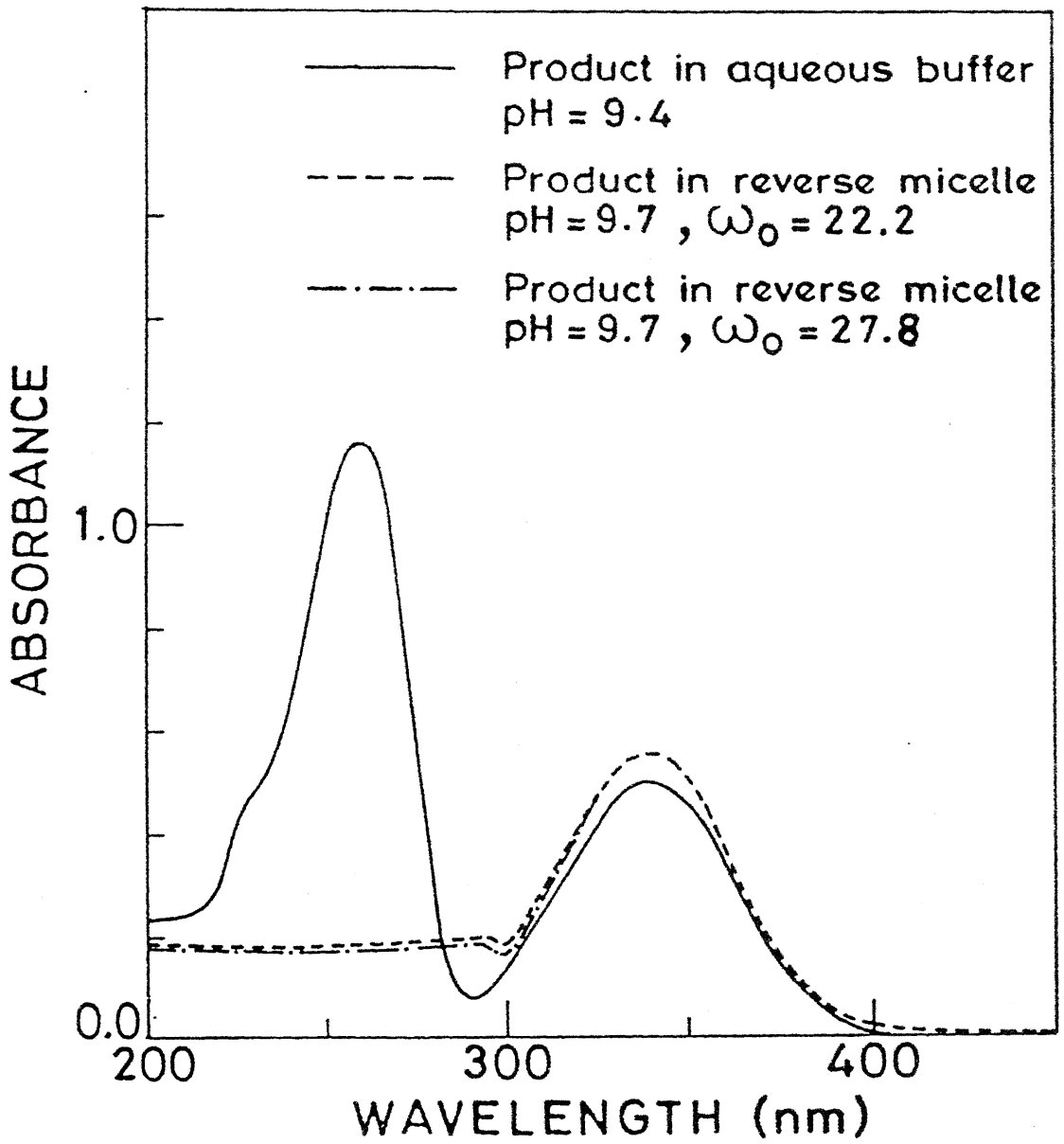


Fig.II.13 Electronic absorption spectra of the product formed (after completion of G-6-PDH reaction) in aqueous buffer (pH = 9.4) and in reverse micellar solution at w_o = 27.8; pH = 9.7 and w_o = 22.2; pH = 9.7. Buffer used was 100 mM glycine-KOH.

NADPH and product (340 nm) remain unchanged in the micellar media. To make sure that the coenzymes are not decomposed in the reverse micellar solution, we extracted out the reverse micelle incorporated coenzymes in 200 mM potassium phosphate buffer, pH = 7.8. The spectra of extracted $NADP^+$, NADPH and product in aqueous buffer showed absorption peaks at 260 nm, 260 nm and 340 nm, 260 nm and 340 nm respectively. This result established beyond doubt that the coenzymes are stable in the reverse micellar solution. The spectra of the product (NADPH) in reverse micelle matches with that of an authentic NADPH sample. Matching of spectrum of product with NADPH (authentic) in reverse micelle indicates that the enzyme G-6-PDH in reverse micellar solution of AOT-Triton X-45-n-heptane catalyzed the reduction of $NADP^+$ to NADPH. It shows that the enzyme in reverse micelle catalyses the same reaction as in aqueous buffer.

In micellar medium the spectra of products (Fig. II.13) recorded at different water pools (w_o=22.2, pH=9.7 and w_o=27.8, pH=9.7) are almost identical. In both the spectra in micellar medium, the peak at 260 nm is missing which is not the case of product in aqueous medium.

II.3.3.5 Time Dependent Stability Study of Glucose-6-Phosphate Dehydrogenase in Reverse Micellar Medium

Stability of enzymes in an environment is of paramount importance because it can improve the storage conditions of enzymes. It has been observed that small amount of water present in the reverse micellar core, can regulate the storage stability

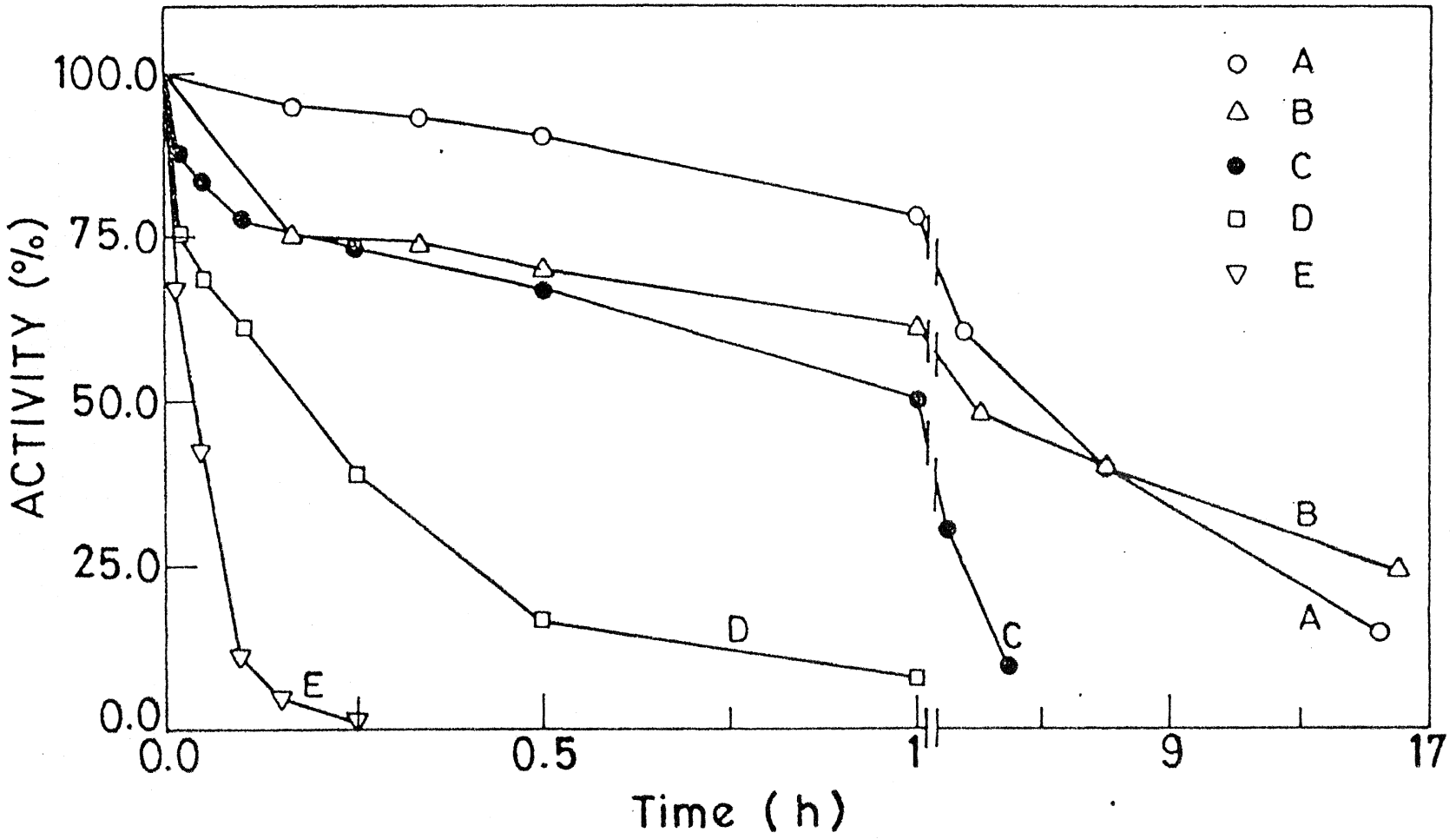


Fig.II.14(a) Stability of G-6-PDH as a function of time in water (100 mM glycine-KOH, pH and 0.1M AOT-0.1M Triton X-45 micellar solution at w_o=27.8, pH=9.7 (100 mM KOH) in presence of substrate, coenzyme. [A] G-6-PDH + $NADP^+$; [B] G-6-PDH (both A and B are in aqueous medium); [C] G-6-PDH + $NADP^+$; [D] G-6-PDH + [E] G-6-PDH (all C, D and E are in micellar media). [G-6-DH] = 0.1 µg/ml, 0.35 mM and [$NADP^+$] = 0.11 mM.

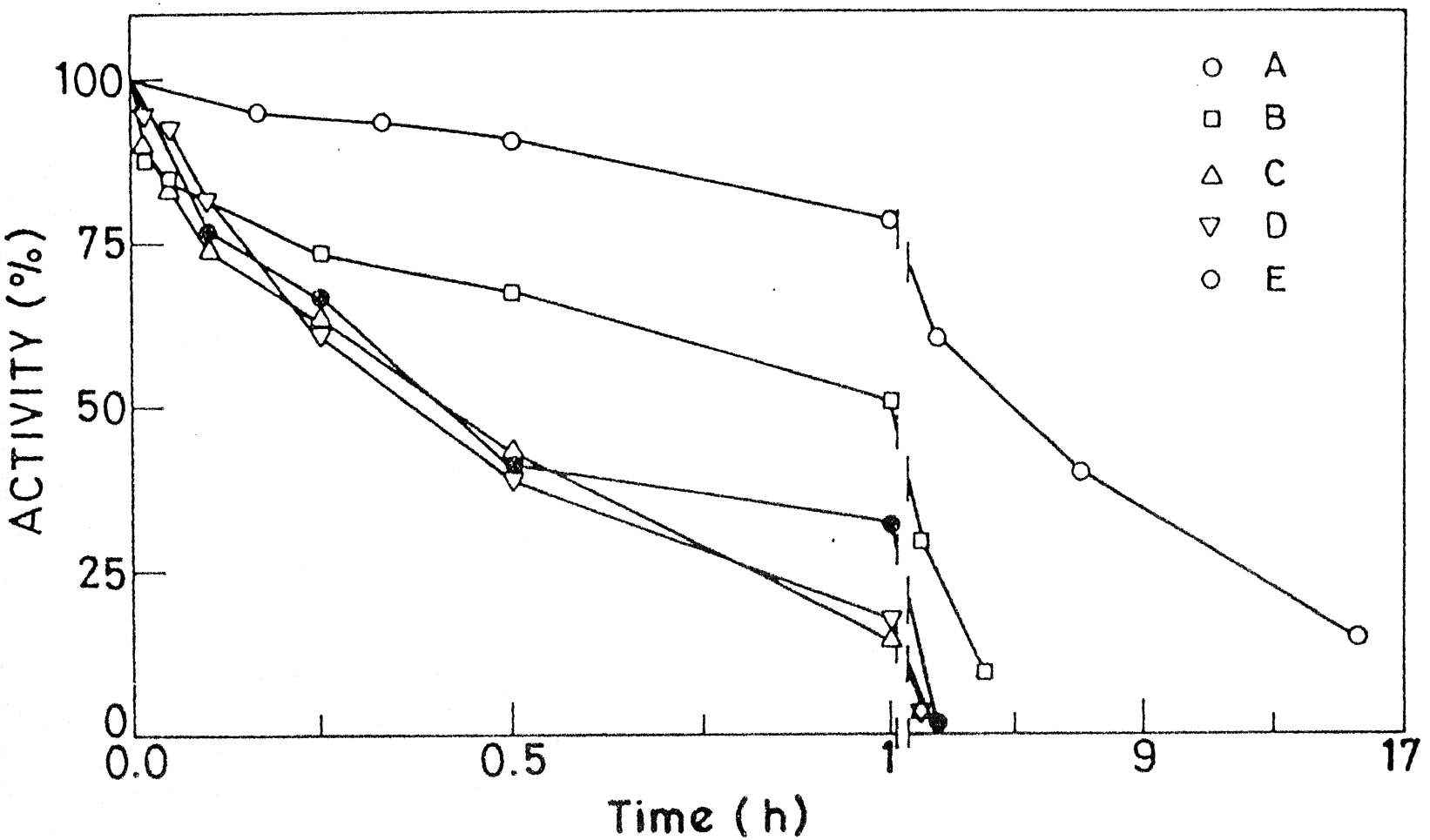


Fig.II.14(b) Stability of G-6-PDH in presence of $NADP^+$ as a function of time in aqueous and in mixed micelles of 0.1M AOT and 0.1M Triton X-45 at different w_o. [A] = water, pH = 9.4; [B] w_o = 27.8, pH = 9.7; [C] w_o = 33.3, pH=9.7; [D] w_o = 38.9, pH = 9.7; [E] w_o = 44.4, pH = 9.7. Buffer used was 100 mM glycine-KOH. Other concentrations were same as in Fig. II.14(a).

of enzyme and the time dependent stability of enzyme in this medium might be better than those stored in aqueous medium.

Figs. II.14 shows the residual activity of G-6-PDH as a function of time. Time dependent stability was found to be dependent on the degree of hydration (w_o) and on the presence of substrate or coenzyme in the storage medium. Fig. II.14(a) shows the change in percentage activity of G-6-PDH as a function of time in aqueous and reverse micelles of AOT-Triton X-45 in n-heptane. The plots have been obtained at different enzyme substrate combinations. Plots A and B give the time dependent stability in aqueous buffer (100 mM Glycine-KOH pH = 9.4). Plot A corresponds to the incubation of G-6-PDH with $NADP^+$ whereas plot B is the result of incubation of G-6-PDH with substrate G-6-P. Similarly plots C,D and E correspond to the incubation of G-6-PDH with $NADP^+$, G-6-PDH with G-6-P and G-6-PDH alone respectively in the reverse micelles of 0.1 M AOT and 0.1 M Triton X-45 in n-heptane (100 mM glycine-KOH) at w_o= 27.8. It is clear from the plots that in both the media, enzyme G-6-PDH incubated with $NADP^+$ is more stable than that of enzyme with glucose-6-phosphate or G-6-PDH alone. In reverse micellar media after about 5 minutes incubation, enzyme alone loses about 80% of its activity, enzyme & G-6-P and enzyme & NADP lose about 35% and 20% activity respectively. On the time scale enzyme alone lost about 80% its activity within 5 minutes whereas enzyme & G-6-P and enzyme & $NADP^+$ lost the same amount of activity after about 1/2 hr. and 5 hrs respectively. The possible reason for the greater stability of G-6-PDH in presence of $NADP^+$ is the formation of strong enzyme-coenzyme complex than enzyme-substrate

complex. These data indicate that coenzyme or substrate improve the stability of enzyme in both aqueous and reverse micellar media. It also shows that incubation of enzyme with $NADP^+$ provides more stable conditions than its incubation with glucose-6-phosphate. Fig. II.14(b) shows the stability data of G-6-PDH at different water pools. In micellar medium maximum stability of enzyme is at w_o=27.8, pH=9.7. The activity of G-6-PDH in 100 mM glycine-KOH buffer pH = 9.4 dropped to approximately 55% after 4 hrs incubation whewreas it was about 10% in micellar medium at w_o = 27.8, pH = 9.7 after same interval of time. The enzyme is more stable in aqueous medium than in reverse micellar medium.

II.3.3.6 Characteristic Constants of Enzymes in Reverse Micelles in Non-aqueous Solvents

The characteristic property of an enzyme is its catalytic property and this aspect of enzymology has excited lots of interest. Any fundamental study of the catalytic function must be based on quantitative measurements of the rate of the catalysed reaction. The initial velocity data can be interpreted to elucidate the mechanism of enzyme reaction. Kinetic studies on enzyme reactions help us comprehend the functioning of enzyme in chemical terms as well as inside the cell.[15,16]

The velocity of an enzyme reaction is influenced by many factors such as enzyme concentration, concentrations of its substrate(s), activators and inhibitors specific for the enzyme, nonspecific effects of compounds (salts and buffers), pH, ionic

strength, temperature etc.[17] Besides these parameters enzyme activity in reverse micellar solution is also dependent on nature of surfactant, surfactant concentration, solvent, water pool size etc. Effect of some of these parameters has already been discussed in earlier part of this chapter. In this section effect of substrate, coenzyme and enzyme concentration will be discussed and the kinetic parameters like K_m, V_{max} etc. will be determined.

II.3.3.6.1 Michaelis-Menten Kinetics

An enzyme catalyzed reaction in general can be satisfactorily analysed by Michaelis-Menten Equation. This equation stipulates that an enzyme (E) combines with a substrate (S) to form an enzyme-substrate complex (ES, also called Michaelis-Menten complex) and the rate of decomposition of the substrate is proportional to the concentration of this complex. At a constant concentration of enzyme, the reaction rate increases with increasing substrate concentration until a maximum velocity is reached. At a sufficiently high substrate concentration, the catalytic sites are filled and so the reaction rate reaches a maximum. Michaelis-Menten proposed the following scheme for the kinetic properties of enzymes.[18,19]

$$E + S \rightleftharpoons ES \xrightarrow{k_{cat}} E + \text{Product} \qquad \ldots (II.1)$$

the catalytic rate is given by

$$V = k_{cat}[ES] \qquad \ldots (II.2)$$

using steady state assumptions, the following Michaelis-Menten equation can be derived

$$V = \frac{k_{cat}[E_o][S]}{K_m+[S]} \quad \ldots (II.3)$$

$$V = \frac{V_{max}[S]}{K_m+[S]} \quad \ldots (II.4)$$

$$\text{where } V_{max} = k_{cat}[E_o];\ K_m = \frac{k_{-1} + k_{cat}}{k_1} \quad \ldots (II.5)$$

K_m is called the Michaelis constant of the substrate. This constant is of great practical importance since it is equal to the substrate concentration at which the reaction rate is half of its maximal value. The Michaelis constant of a substrate is, therefore, a measure of the affinity of the enzyme for the substrate. The lower the constant, the higher the affinity.

The Michaelis-Menten equation (II.4) is converted into linear form of double reciprocal or Lineweaver-Burk plot.

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} \quad \ldots (II.6)$$

plot of 1/V against 1/[S] gives a straight line with an intercept of $1/V_{max}$ and slope of K_m/V_{max}. From equation (II.6) the value of K_m and V_{max} can be obtained graphically. In the present study the kinetic parameters have been determined from Lineweaver-Burk plots.[18,19]

II.3.3.6.2 Enzymic Reaction in Reverse Micellar Systems

The kinetic theory for aqueous solutions of surfactants is also found to be valid for systems with reverse micelles.[20] This theory has been extended for a reaction between enzyme E and substrate S which obeys the Michaelis-Menten kinetics in an 'organic solvent-water-surfactant' system.

$$E + S \rightleftharpoons ES \longrightarrow E + \text{Products} \quad \ldots (II.7)$$

There are assumptions

(i) a solution of a surfactant consists of two phases[21], i.e. a bulk 'phase' of an organic solvent and a 'phase' of micelles wetted by water.

(ii) the substrate is distributed evenly between the two phases.[22]

$$(S)_{\text{in the bulk phase}} \rightleftharpoons (S)_{\text{in micelles}} \quad \ldots (II.8)$$

The partition coefficient is expressed in the following way:

$$P_s = \frac{[s]_{micelle}}{[s]_{bulk}} \quad \ldots (II.9)$$

We need not take into account the distribution of enzyme into two media as proteins are practically insoluble in hydrophobic solvents.[23] Moreover in non-aqueous media enzymes usually get denatured.[24] Therefore, it is assumed that all the catalycic activity is restricted to the micellar phase.

During the initial time (when concentration of products is negligible compare to substrate concentration) with excess of the substrate over the enzyme and under stesdy-state conditions, the reaction rate of Eqn. II.7 in the form of Michaelis-Menten equation can be expressed as

$$v = \frac{k_{cat,micelle}[E_o]_{micelle}[S_o]_{micelle}}{K_{m,micelle} + [S_o]_{micelle}} \cdot Q \qquad \ldots (II.10)$$

It is assumed that the exchange of the substrate molecules between the phases is sufficiently fast i.e. enzyme reaction (II.7) does not violate equilibrium (II.8) and equation (II.9) is only valid for sufficiently dilute solutions i.e. the concentration of the reagents should be much lower than that of the surfactants.

Then the concentration of the reagents are

$$[S_o]_{total} = [S_o]_{micelle} \cdot Q = [S_o]_{bulk} (1-Q) \qquad \ldots (II.11$$

and $$[E_o]_{total} = [E_o]_{micelle} \cdot Q \qquad \ldots (II.12$$

On substituting equations (II.9), (II.11) and (II.12) into eqn. (II.10) we get

$$v = \frac{k_{cat,apparent}[E_o]_{total} \cdot [S_o]_{total}}{K_{m,apparent} + [S_o]_{total}} \qquad \ldots (II.1$$

when $k_{cat,apparent} = k_{cat,micelle}$

$$\text{and } K_{m,\text{apparent}} = K_{m,\text{micelle}} \frac{1 + Q(P_s - 1)}{P_s}$$

If one assumes in the case of a charged substrate that the substrates are confined to aqueous micellar phase i.e. $P_s >> 1$ and $P_s Q >> 1$ then

$$K_{m,\text{apparent}} = K_{m,\text{micelle}} \cdot Q \qquad \ldots (II.14)$$

It was pointed out by Luisi et al.[25] that the concentration of reactants, substrates and enzyme in the reverse micellar solution can be expressed in two different ways. Depending on whether one operates with water pool (wp) concentrations or with overall (ov) concentrations there will be two K_m values namely $(K_m)_{ov}$ and $(K_m)_{wp}$ which are numerically related by the factor f

$$(K_m)_{ov} = (K_m)_{wp} . f \qquad \ldots (II.15)$$

where $$f = F_w + P\,(1 - F_w) \qquad \ldots (II.16)$$

F_w is the water volume fraction and P_s represents the partition coefficient of the substrate (the enzyme is assumed to be soluble only in water pools).

$$\text{when } P = 1,\ (K_m)_{wp} = (K_m)_{ov} \qquad \ldots (II.17$$

For substrates that are preferentially soluble in the water pool i.e. $P < 1$.

Hence $(K_m)_{wp} > (K_m)_{ov}$ ... (II.18)

and when P=0 i.e. reagent that is only soluble in water pool

$$(K_m)_{ov} = (K_m)_{wp} \cdot F_w \quad \text{... (II.19)}$$

Since K_m is a good measure of the dissociation constant of the enzyme substrate (ES) complex, therefore it becomes all the more necessary to decide that which K_m[either $(K_m)_{wp}$ or $(K_m)_{ov}$] is the physically relevant one. In general for most of the enzymes it is found that the value of $(K_m)_{ov}$ is more close to $(K_m)_{aqueous}$ than $(K_m)_{wp}$.

II.3.3.6.3 Effect of Enzyme Concentration on Enzyme Reaction Velocity

If enzyme molecules are acting independently in solution than the enzyme reaction velocity should be proportional to enzyme concentration. Effect of enzyme concentration also studied on reaction velocity give information about the activator effect, sub-unit association and presence of any toxic impurity. The plot of initial velocity of enzyme reaction as a function of enzyme concentration in reverse micelle is plotted in Fig. II.15. The variation is found to be linear. This is one of the indirect proof that the change in absorbance in reverse micellar solution is due to the formation of product. As we go on increasing the concentration of enzyme, velocity of reaction which is independent of enzyme moloecules increases linearly.

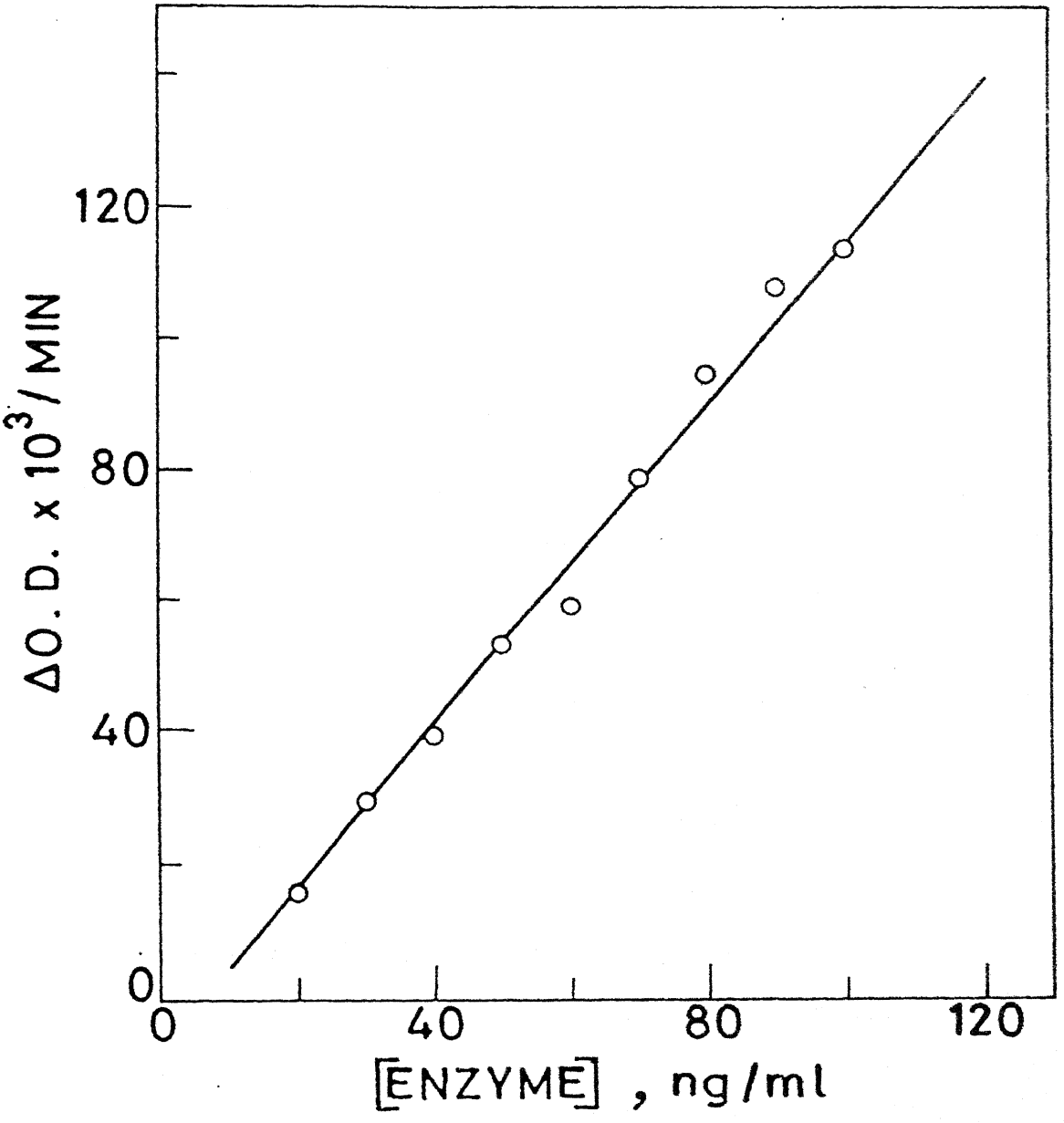


Fig.II.15 Dependence of initial velocity of enzyme reaction on G-6-PDH concentrations in 0.1M AOT-0.1M Triton X-45-n-heptane reverse micellar system at w_o = 27.8, pH = 9.7. The concentrations were [G-6-P] = 0.35 mM, [$NADP^+$] = 0.11 mM. Buffer was 100 mM glycine-KOH.

II.3.3.6.4 Effect of Substrate and Coenzyme Concentrations on Reaction Velocity

In reverse micellar solution study on the dependence of the enzyme reaction rate with substrate and enzyme concentrations shows that the glucose-6-phosphate dehydrogenase follows Michaelis-Menten kinetics. Effect of $NADP^+$ concentration and G-6-P concentration separately (keeping concentration of other component constant) is shown in Figs. II.16 & II.17. The initial velocity of the reaction initially increases linearly and then reaches a maximum. With further increase of $NADP^+$ the reaction velocity remains at its saturation value. However in the case of G-6-P, the velocity reaches a maximum and then shows a decrease with increase of G-6-P which is indicative of inhibition. In reverse micellar solution initial velocity of the enzyme shows maximum at glucose-6-phosphate and $NADP^+$ concentrations of 400 μM & 80 μM respectively. Whereas in aqueous buffer the saturation velocity was obtained at concentrations 220 μM and 25 μM respectively.

At high G-6-P concentration the deviation from Michaelis-Menten kinetics occurs because of substrate inihibition. Due to this substrate inhibition hyperbolic kinetics with an asymptote V_{max} may not apply.[17] When initial velocity of enzyme reaction is plotted against concentration of G-6-P, maximum velocity is obtained at 400 μM and at concentrations beyond 400 μM, G-6-P shows substrate inhibition. This phenomenon of substrate inhibition may be due to formation of tight substrate-NADPH-enzyme abortive complex.

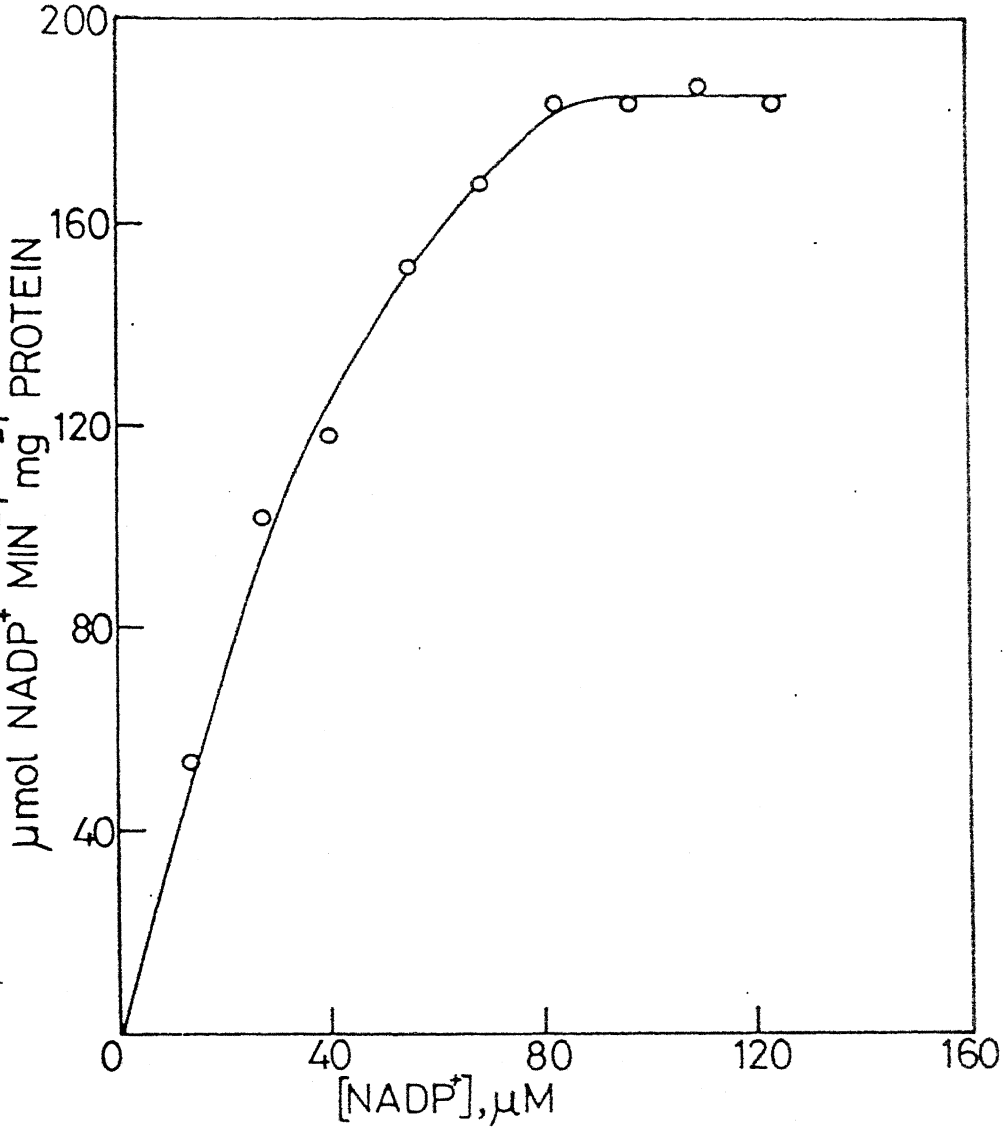


Fig.II.16 Dependence of G-6-PDH activity on $NADP^+$ concentrations in 0.1M AOT and 0.1M Triton X-45 mixture in n-heptane at w_o = 27.8 and pH = 9.7. The concentration of G-6-P was 0.35 mM and buffer was 100 mM glycine-KOH.

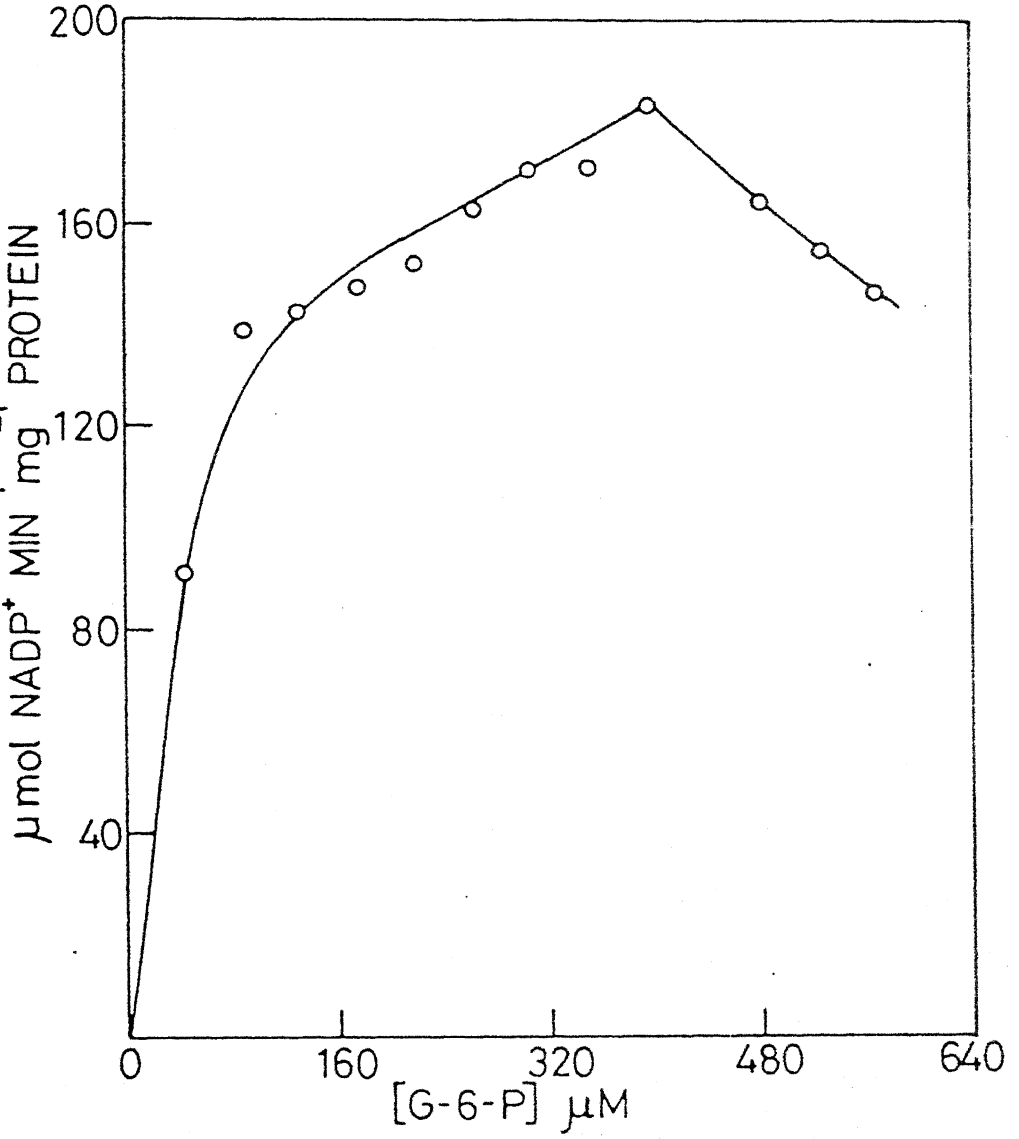


Fig.II.17 Effect of G-6-P concentrations on G-6-PDH activity in 0.1M AOT and 0.1M Triton X-45 mixture in n-heptane at w_o = 27.8 and pH 9.7. The concentration of $NADP^+$ was 8.11 mM and buffer was 100 mM glycine-KOH.

II.3.3.6.5 Double Reciprocal Plots: Determination of Kinetic and Binding Parameters of Glucose-6-phosphate Dehydrogenase in Reverse Micelles

Variation of inverse of G-6-DPH catalyzed reaction rate with inverse of $NADP^+$ concentrations at four fixed concentrations of G-6-P is shown in Fig. II.18. These plots are linear and meet on x-axis. Data for the primary plots of Fig. II.18 & II.19 were obtained by observing the effect of substrate and coenzyme concentrations on G-6-PDH activity at w_o= 27.8, pH = 9.7 in 0.1M AOT and 0.1 M Triton X-100 (1:1) in n-heptane. The Michaelis const (K_m) for $NADP^+$ and G-6-P were determined from the secondary plots obtained by plotting intercept on 1/v axis of one substrates against reciprocal of concentration of other substrate (Figs. II.20 & II.21. The values are summarized in Table II.1.

Comparison of data in Table II.1 shows that reverse micelles $(K_m)_{ov}$ for $NADP^+$ increases 16.1 times and $(K_m)_{ov}$ for G-6-P increase 3.4 times in comparison to their values in aqueous medium. On the other hand $(K_m)_{wp}$ of $NADP^+$ is 161 times and $(K_m)_{wp}$ of G-6-P is 34.5 times of their values in aqueous medium. $(K_m)_{ov}$ in reverse micellar solution is taken as the valid one since reverse micellar solution behaves as a homogeneous solution and therefore substrate concentration is considered for overall volume. Increase in $(K_m)_{ov}$ signifies that enzyme-substrate complex is significantly destabilised as compared to that in aqueous buffer.

Our results demonstrate that the water pool size extensively regulates the catalytic activity of enzyme G-6-PDH entrapped in

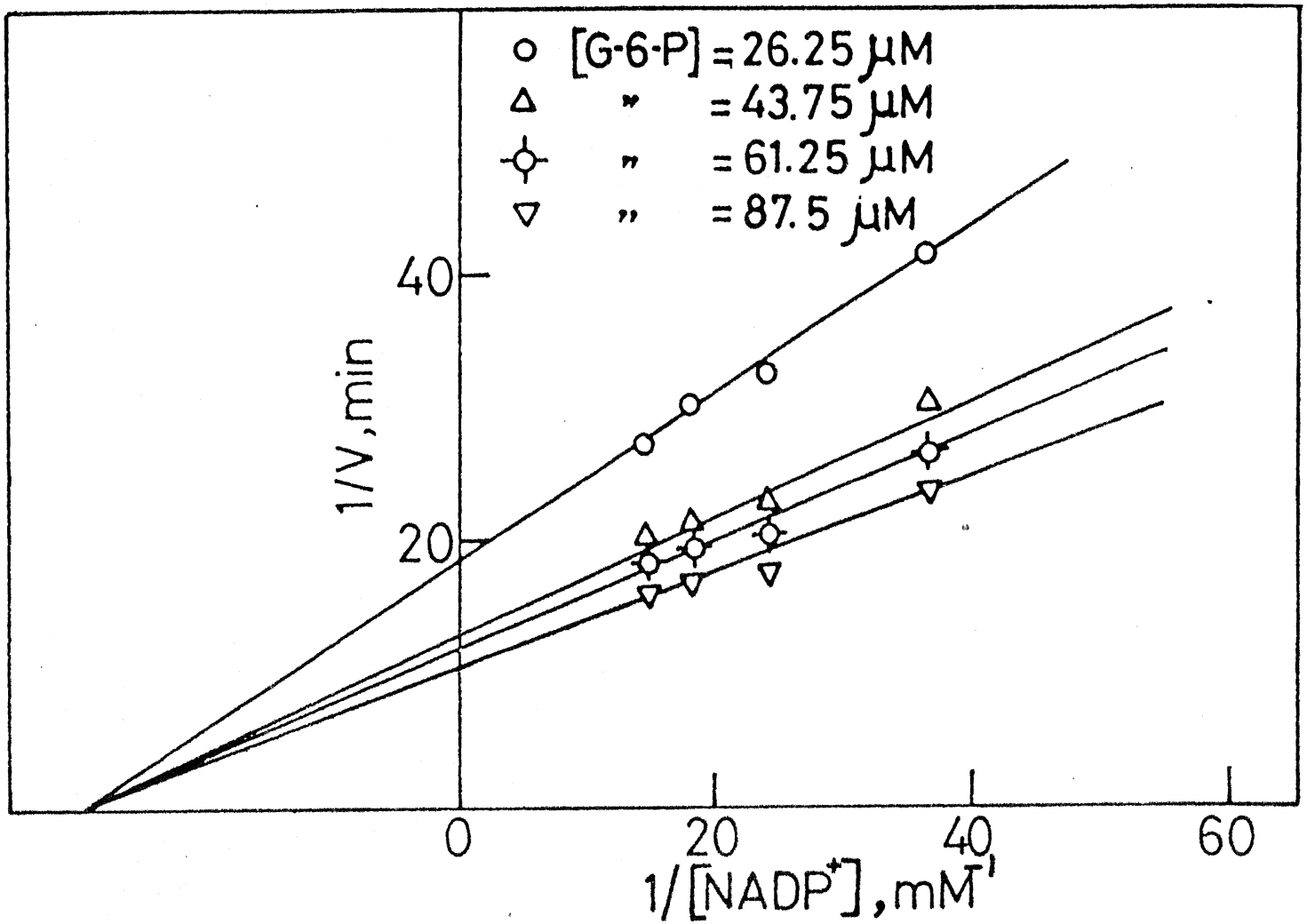


Fig.II.18 Lineweaver-Burk plots for initial G-6-PDH rate with $NADP^+$ concentrations in 0.1M AOT and 0.1M Triton X-45 in n-heptane at different G-6-P concentrations at w_o = 27.8 and pH = 9.7. Buffer was 100 mM glycine-KOH.

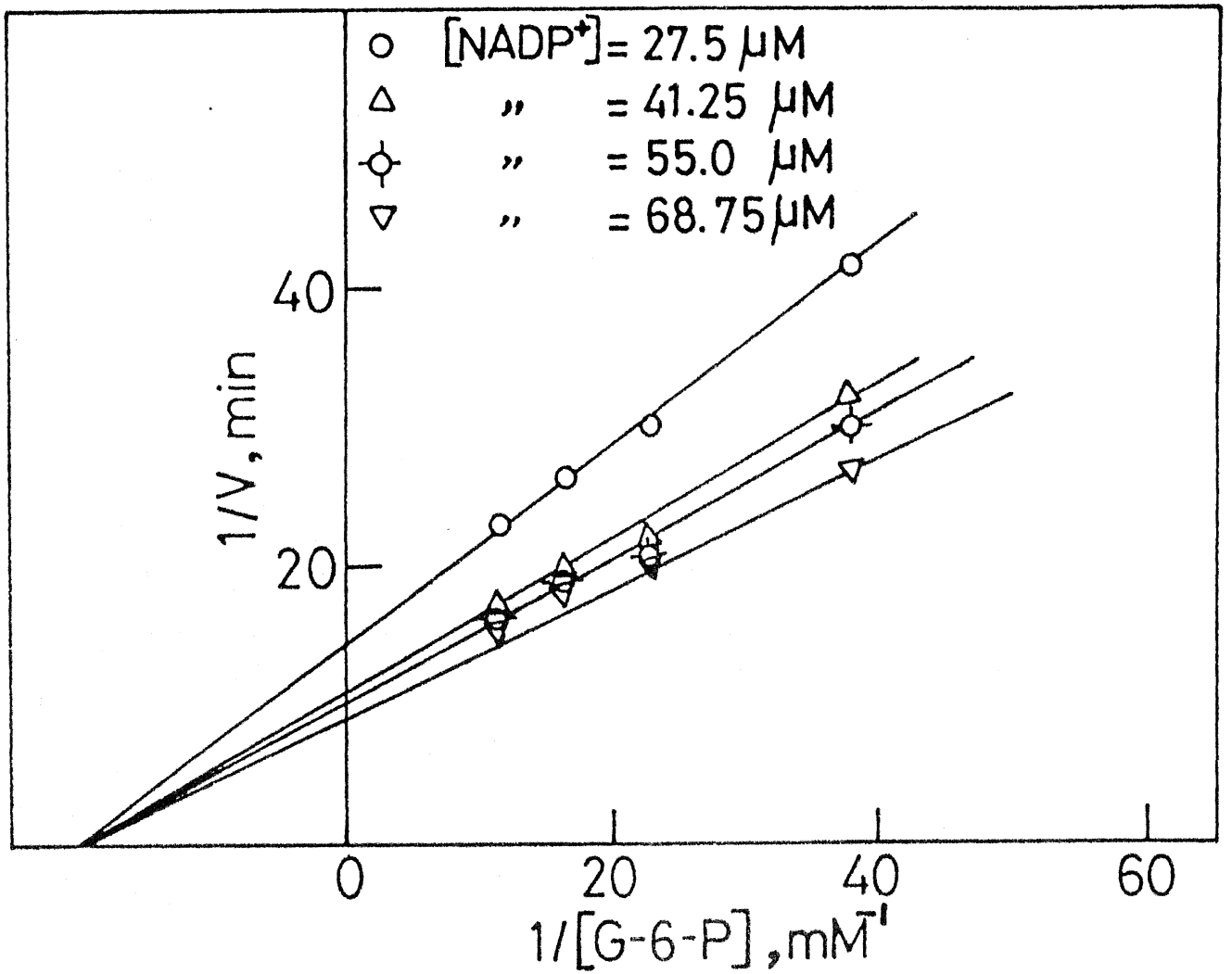


Fig.II.19 Double reciprocal plots for initial G-6-PDH rate with G-6-P concentrations in 0.1M AOT and 0.1M Triton X-45 mixture in n-heptane at different $NADP^+$ concentrations and w_o = 27.8, pH = 9.7. Buffer was 100 mM glycine-KOH.

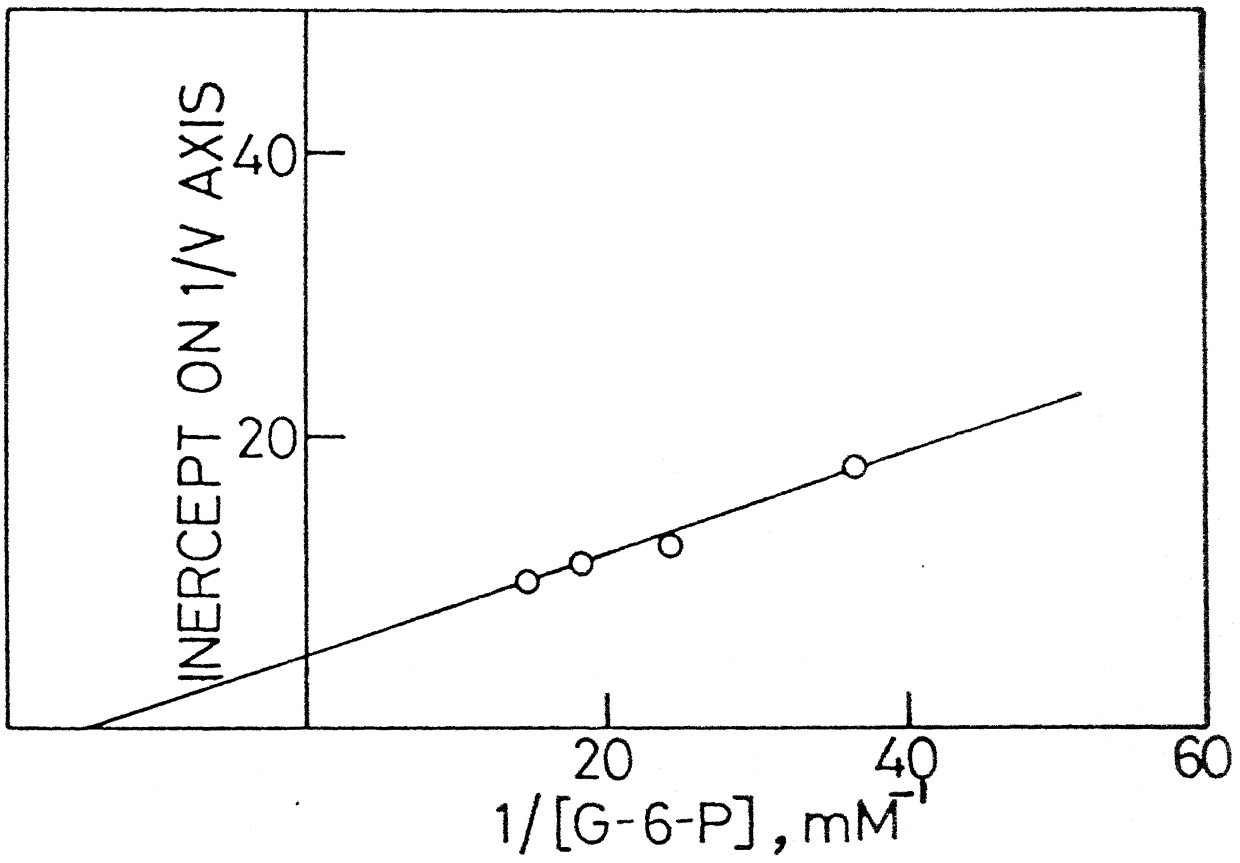


Fig.II.20 Intercepts on 1/v axis of Fig. II.18 is plotted as a function of inverse of G-6-P concentrations.

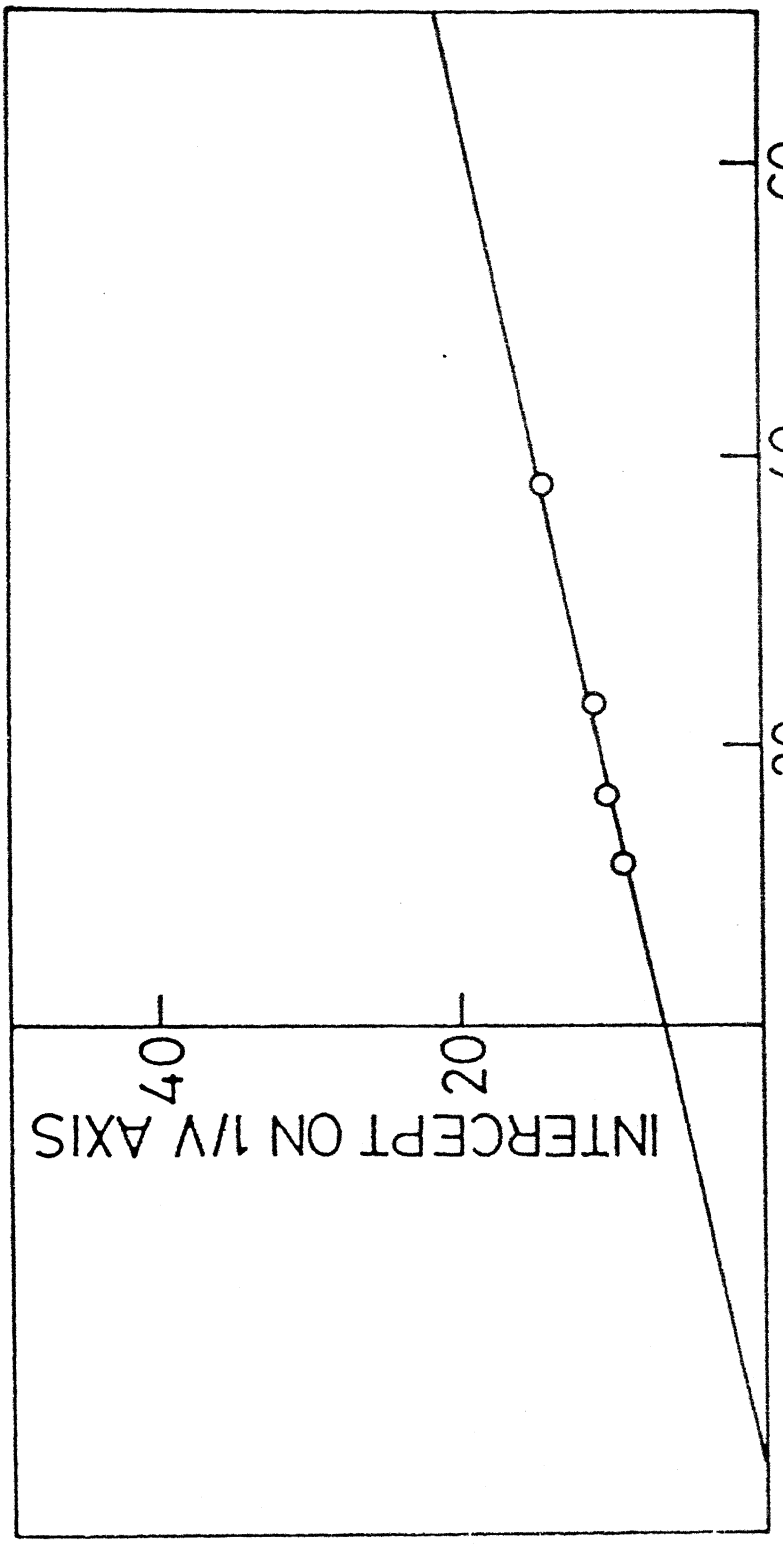


Fig.II.21 Plot of intercepts on 1/v axis of Fig. II.19 with reciprocal of $NADP^+$ concentrations.

TABLE II.1 Characteristic Constants of Glucose-6-phosphate Dehydrogenase

	0.1M AOT and 0.1M Triton X-45 mixture in n-heptane, w_o=27.8 (pH=9.7, 100 mM glycine-KOH	Standard Deviation	Tris Buffer pH = 8.0, 38°C, 10^{-2}M $MgCl_2$	
$(K_m^{NADP})_{ov}$, M	3.22×10^{-5}	0.226×10^{-5}	2.0×10^{-6}	(26)
$(K_m^{NADP})_{wp}$, M	3.22×10^{-4}	-	-	
$(K_m^{G-6-P})_{ov}$, M	6.896×10^{-5}	0.898×10^{-5}	2.0×10^{-5}	(26)
$(K_m^{G-6-P})_{wp}$, M	6.890×10^{-4}	-	-	
$(K_d^{NADP})_{ov}$, M	3.478×10^{-5}	-	-	
$(K_d^{NADP})_{wp}$, M	3.475×10^{-4}	-	-	
V_{max}, μ mol $l^{-1}min^{-1}$ (mgenzyme)$^{-1}$	183.900	-	133.37 (100 mM glycine-KOH, pH=9.4)	

ov = Overall

wp = Water pool

the reverse micelles. Loss of enzyme activity at lower values of w_o (w_o less than 25) may be attributed to the small water pool size where big molecules of G-6-PDH may not be able to acquire functional conformation due to volume squeeze or due to distortion of the active site of enzyme. The optimum value of w_o probably depends on the size and complexity of the enzyme. More than one $w_{o,opt}$ is possible, if the enzyme changes its polymeric form (dimer or tetramer) with variation of water pool. Apparently the enzyme shows maximum activity when the size of water pool inside the reverse micelle is such that it suitably accomodates the enzyme in its most active conformation. The super activity of the enzyme G-6-PDH, is a rare phenomenon which may be attributed to the property of the water pool in the microcaptive environment of reverse micelles.

Small difference (~ 0.2 units) in optimum pH in aqueous and reverse micelles in the case of G-6-DH indicates that even in the micellar microenvironment at condition for maximum activity, pK_a of amino acid residues remains almost unchanged.

When we change ratio of surfactant concentration it means that we are approaching to a reverse micellar solution of one single surfactant from other surfactant. In either of the surfactants the enzyme is less active and the combination of equal amounts of the surfactant provide the system suitable environment for good activity.

Variation of total surfactant concentrations at fixed percentage of water (surfactant ratio is one) changes water pool size and therefore the nature of the enzyme activity vs

surfactant concentration curve should be the same as that of activity vs w_o profile.

However when we change total surfactant concentration keeping w_o and ratio of two surfactant concentrations (1:1) constant, we observe an interesting phenomenon. The enzyme shows super activity in the range 0.1M - 0.18 M total surfactant concentrations. Equal amount of two surfactants is a critical ratio and an absolute requirement for maximum activity of the enzyme. At low concentrations of total surfactant the enzyme molecules are not well protected from the unfavourable action of organic solvent.

The masking of absorption maxima at 260 nm for coenzymes $NADP^+$ and NADPH in mixed reverse micelles indicate that probably these coenzymes directly interact with the surface of the reverse micelles. Our spectral study implies that though the enzyme is catalysing the same reaction as in aqueous medium, the micro-environment around coenzymes inside reverse micelle is different from that of aqueous buffer.

Since reverse micelles have some features similar to those of biomembranes, therefore display of super activity of G-6-PDH shows that reverse micelles constitute more realistic model for *in vitro* study of enzymes in comparison with classical *in vitro* experiments in aqueous buffers.

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CHAPTER III

PROPERTIES AND CHARACTERISTICS OF ALCOHOL DEHYDROGENASE IN ANIONIC REVERSE MICELLAR MEDIA

III.1 INTRODUCTION

In vitro study of enzymatic reactions in reverse micelles of surfactants in organic solvents[1] opens up possibilities of regulating enzymatic activity by changing the composition and physicochemical characteristics of the reverse micelles. This novel reaction medium offers tremendous potential for the use of enzymes in different fields. For example enzymes in such media have found application in fine organic syntheses,[2-5] in clinical and chemical analyses,[6-9] in medicine[10-13] and in protein chemistry[14-17] etc. As there is no apparent generalization of enzymic behavior in reverse micelles, it becomes necessary to investigate their functional properties in reverse micelles individually.

To understand the behavior of enzymes in reverse micelles in non-aqueous solvents another oxidoreductase viz. alcohol dehydrogenase (alcohol:NAD^+ oxidoreductase, EC 1.1.1.1, ADH(Y)) has been chosen. It is a multiunit (4 sub-unit) enzyme and is

involved in metabolic mechanism in the living cells and plays very specific roles in other biological processes. A thorough study of the properties and functions of an enzyme with a quaternary structure in reverse micelles provides the possibility of learning about the role of intersub-unit and protein-protein interactions. Alcohol dehydrogenase from yeast is a complex enzyme having M.W. ~ 151,000 dalton with 4 subunits. It catalyzes the oxidation of alcohols and the reduction of aldehydes and ketones in the presence of NAD^+ and NADH respectively.

$$RCH_2OH + NAD^+ \rightleftharpoons RCHO + NADH + H^+$$

Yeast alcohol dehydrogenase has a more narrow specificity than does the horse liver enzyme. ADH(Y) accepts ethanol as substrate. It is less active on other straight chain primary alcohols, and acts to a very limited extent on certain secondary and branched chain alcohols. $NADP^+$ does not serve as coenzyme. The common reaction in yeast cells and in several other organisms is the reduction of acetaldehyde to ethanol by ADH(Y). It is used analytically for the determination of ethanol and as a coupling enzyme for other reactions which yield ethanol, such as the hydrolysis of benzonyl-1-arginine ethyl ester (BAEE).

For the study of ADH(Y) in reverse micelle, the system anionic surfactant sodium bis(2-ethylhexyl)sulfosuccinate abbreviated as Aerosol OT or AOT in isooctane has been used. The characteristic property of this reverse micellar system is that it takes in large amount of water which is needed to solubilize a

relatively big enzyme like yeast alcohol dehydrogenase. A detailed study on the solubilization and kinetic characteristies of yeast alcohol dehydrogenase in the reverse micellar solution of AOT/Isooctane has been presented in this chapter.

III.2 EXPERIMENTAL SECTION

III.2.1 Materials

Yeast alcohol dehydrogenase (EC 1.1.1.1) was purchased from Boehringer Mannheim, USA as a crystalline suspension in ammonium sulfate solution, 3.2 mol/l, pH approx. 6. The enzyme was used as such with proper dilution in pyrophosphate buffer (10 mM, pH = 8.3). NAD^+ was obtained from Sigma Chemical Co., USA. Ethyl alcohol used was purified by usual method.

Sodium bis(2-ethylhexyl)sulfosuccinate (AOT) was obtained from Cynamid Company, USA. It was purified by the reported method.[18] The purified AOT was made into small pieces and then dried for 12 hrs in vacuum desiccator over P_2O_5 prior to use. Isooctane obtained from Fluka, Switzerland was of puriss grade.

Sodium pyrophosphate buffer solution and buffer component glycine was purchased from Sigma Chemical Co., USA. Potassium hydroxide used for buffer preparation was from BDH, England. All buffer solutions were made in double distilled water.

III.2.2 Preparation of Reverse Micellar Solution Containing and Substrate

Reverse micellar solution of AOT in isooctane was prepar

by dissolving required amount of dry AOT in isooctane. Injection method[19] has been used to prepare homogeneous and optically transparent solution of enzyme and substrates in the reverse micelles. C_2H_5OH, NAD^+ and buffer solutions were solubilized by injecting small aliquots of 2-30 μl of the solution into AOT/isooctane from Hamilton microliter syringes. The solution became clear by vortexing it for 5-15 secs. Ethyl alcohol was always added first as it quickens clarification of the solution. Pyrophosphate buffer (10 mM, pH = 8.3) was used to prepare the stock solutions of enzymes. Coenzyme and substrate solutions were made in glycine-KOH buffer. pH of the buffer solutions were measured on ELICO LI-120 digital pH-meter at 30°C. Enzyme, coenzyme, substrate and buffer concentrations were adjusted according to the ease of solubilization at specified water pools.

III.2.3 Assay of Alcohol Dehydrogenase Activity

Enzyme activity measurements were done spectrophotometrically on a Gilford model 260 UV/Vis spectrophotometer at 30°C as described in chapter II. In this case the kinetic run was monitored by recording the increase of absorbance at 340 nm for NADH formation. Calculation of enzyme activity was done as mentioned in chapter II. One unit of alcohol dehydrogenase reduces 1 μmole of NAD^+ per minute. Specific activity is defined as the number of units per milligram of protein.

where $A_{340\ nm}$/min is change in absorbance per minute at 340 nm for NADH formation.

III.3 RESULTS AND DISCUSSION

Reverse micellar solution with a small amount of water has been used for solubilization and study of enzymes in vitro. Enzymes are encapsulated in the water inside the reverse micelles and thus avoid denaturing effects of organic solvent and surfactant molecules. The water pools behave as novel microreactors where enzymes are made to work in the microcaptive environment.

III.3.1 Solubilization of Alcohol Dehydrogenase in Reverse Micelles

We have successfully solubilized yeast alcohol dehydrogenease ADH(Y) in AOT/isooctane reverse micellar solution. This water soluble enzyme forms a homogeneous and optically transparent solution in the reverse micelles. It indicates that this hydrophilic enzyme is solubilized in the reverse micelles according to water-shell model. In this model a shell of water molecules around the enzyme inside the water pool of the reverse micelles helps the enzyme avoid harmful contact with surfactant wall and bulk organic solvent. A small fraction of the micelles are expected to be occupied by enzyme molecules, the rest being available for coenzyme and substrate. These occupied and unoccupied micelles are considered to be in dynamic equilibrium.

Solubilization of the enzyme in the reverse micellar solution of AOT/isooctane surfactant was found to be dependent on surfactant concentration, the molar ratio of water to surfactant (w_o), pH and concentration of buffers and temperature etc. The overall concentration has been considered throughout unless stated otherwise in the present study. The concentration of aqueous stock solution of enzyme and salt concentration in which enzyme is stored as a suspension, was found to affect the solubility of enzymes in reverse micelles significantly. Commercially supplied stock solution of ADH(Y) was diluted to 1500 times to adjust the initial velocity of enzyme reaction in a better measureable range. The concentration of aqueous stock solution of ADH(Y) to be solubilised in the reverse micelle was 0.02 mg/ml. 100 mM glycine-KOH buffer was found to be suitable for the present study. The pyrophosphate buffer in which assay is carried out in aqueous medium is not suitable for the assay conditions in the reverse micelle.

III.3.2 Enzyme Activity in Reverse Micellar Solution

To optimize the conditions for maximum activity of ADH(Y) in the reverse micellar solution of AOT/isooctane we have systematically studied the effect of water pool, pH, and surfactant concentration on enzyme activity.

III.3.3.1 Effect of Degree of Hydration (w_o) on Enzyme Activity

The properties of the water pool inside the reverse micelle are dictated by its size. One of the most striking feature

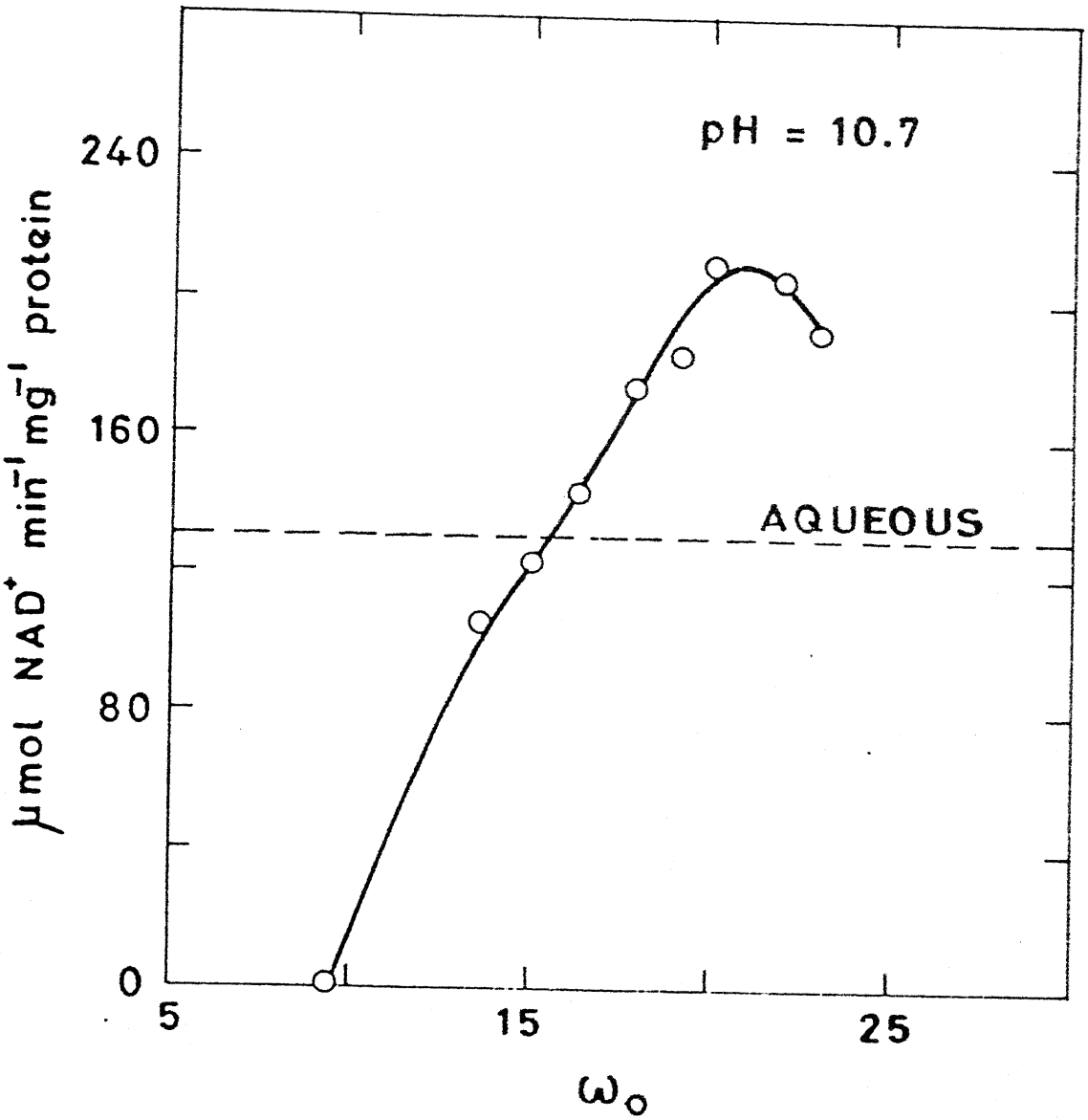

Fig.III.1 Specific activity of ADH(Y) in AOT-isooctane reverse micelles as a function of w_o. The concentrations were [AOT] = 100 mM; $[NAD^+]^o$ = 0.5 mM and $[C_2H_5OH]$ = 100 mM. Buffer was 100 mM glycine-KOH. [ADH(Y)] = 0.08 µg/ml.

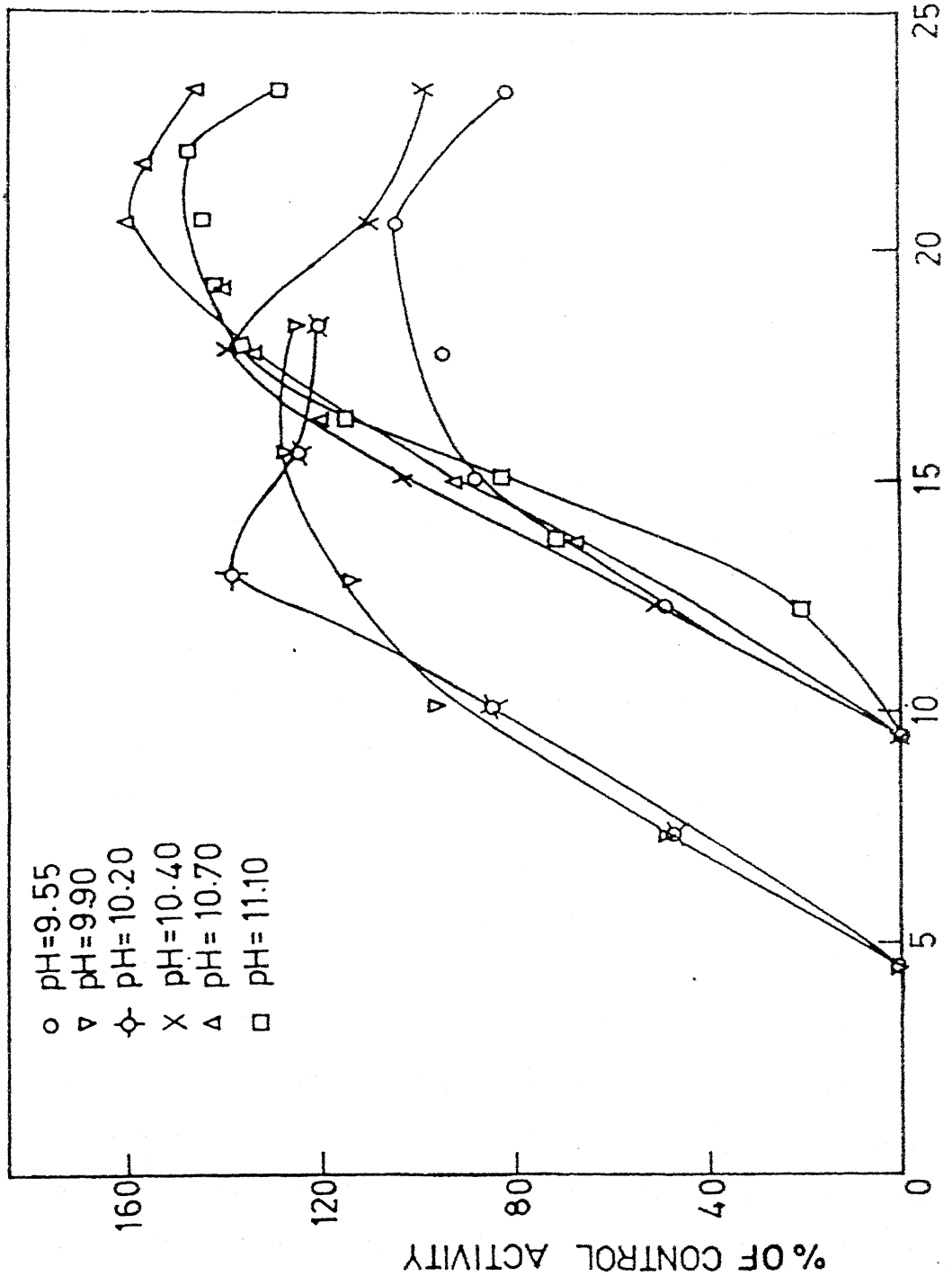

Fig.III.2 Percentage control activity of ADH(Y) in AOT-isooctane reverse micelles as a function of w_o. The concentrations were [AOT] = 100 mM; [NAD^+] = 0.5 mM and [C_2H_5OH] = 100 mM. Buffer was 100 mM glycine-KOH. [For pH 9.90 and 10.2 the w_o-axis is shifted left by 5 units for clarity].

observed in the study of properties of enzymes in reverse micelles is the dependence of catalytic activity of solubilized enzymes on the degree of hydration (w_o) of the reverse micelles. The specific activity of the enzyme with the change of degree of hydration at pH = 10.7 has been shown in Fig. III.1. Fig. III.2 shows the plot of percentage control activity versus water pool at different pH in the range 9.55 to 11.1. The size of the water pool can be varied either by regulating water concentration or surfactant concentration and the molar ratio of water to surfactant concentration is referred to as water pool (w_o). We have changed water pool size by varying the water content in the reverse micelles. The activity of the enzyme increases with increase of water pool size, reached a maximum and then decreases at every pH studied. This kind of bell-shaped plot was obtained in earlier cases like α-chymotrypsin[20] and recently studied enzymes malate[21] dehydrogenase and dihydrofolate reductase.[22] The bell- shaped dependence of activity upon the magnitude of w_o seems to be a general trend in micellar enzymology. At all pH values the maximum activity was obtained at around w_o = 20. The most striking effect is that below the water pool 9.4 there is no activity at all and with increase of water pool the activity showed a sharp increase. Even at the highest water pool the activity though was less than the maximum value, yet it was almost 100%. It is probable that at water pool less than 10. the size of the water pool is such that it can not accommodate the relatively big enzyme ADH(Y) having four sub-units in proper conformation and it is in such a squeezed condition that its active sites are not functional. With the increase in water pool

size it starts gaining its activity sharply and ultimately manifests its full activity. At intermediate water pools w_o = 17-21, the property of the water inside the water pool i.e. its microviscosity, dielectric constant etc. helps the enzyme to assume its most viable conformation. It shows superactivity in this range of water pool. The term super activity means, that the activity of the enzyme is more than its activity in aqueous buffer at optimal conditions. The maximum activity obtained with change of water pool was 159.77% that of control at w_o = 20.6 and pH = 10.7. Here maximum activity was obtained at higher water pools and it is in line with enzymes like malate dehydrogenase,[21] glutathione reductase[23] and dihydrofolate reductase[22] though the same for α-chymotrypsin[20] was obtained at low water pools. The decrease in activity after the maximum may be due to decrease in interfacial tension inside the reverse micelle. Decrease in interfacial tension causes the enzyme to be exposed to harmful contact of organic solvent.

III.3.3.2 Effect of pH on Enzyme Activity

Specific activity - pH profile for the enzyme ADH(Y) in aqueous and reverse micellar solution is shown in Fig. III.3. The variations of percentage control activity with pH are shown in Fig.III.4 The activity increases with increase in pH and then again decreases. The bell shaped curve is similar in shape to that obtained in aqueous buffer. In aqueous medium, the maximum activity was at pH = 8.3 (pyrophosphate buffer, 10 mM) whereas in reverse micellar medium maximum activity occurred at pH 10.7 which is 2.4 units higher than that in aqueous solution. This

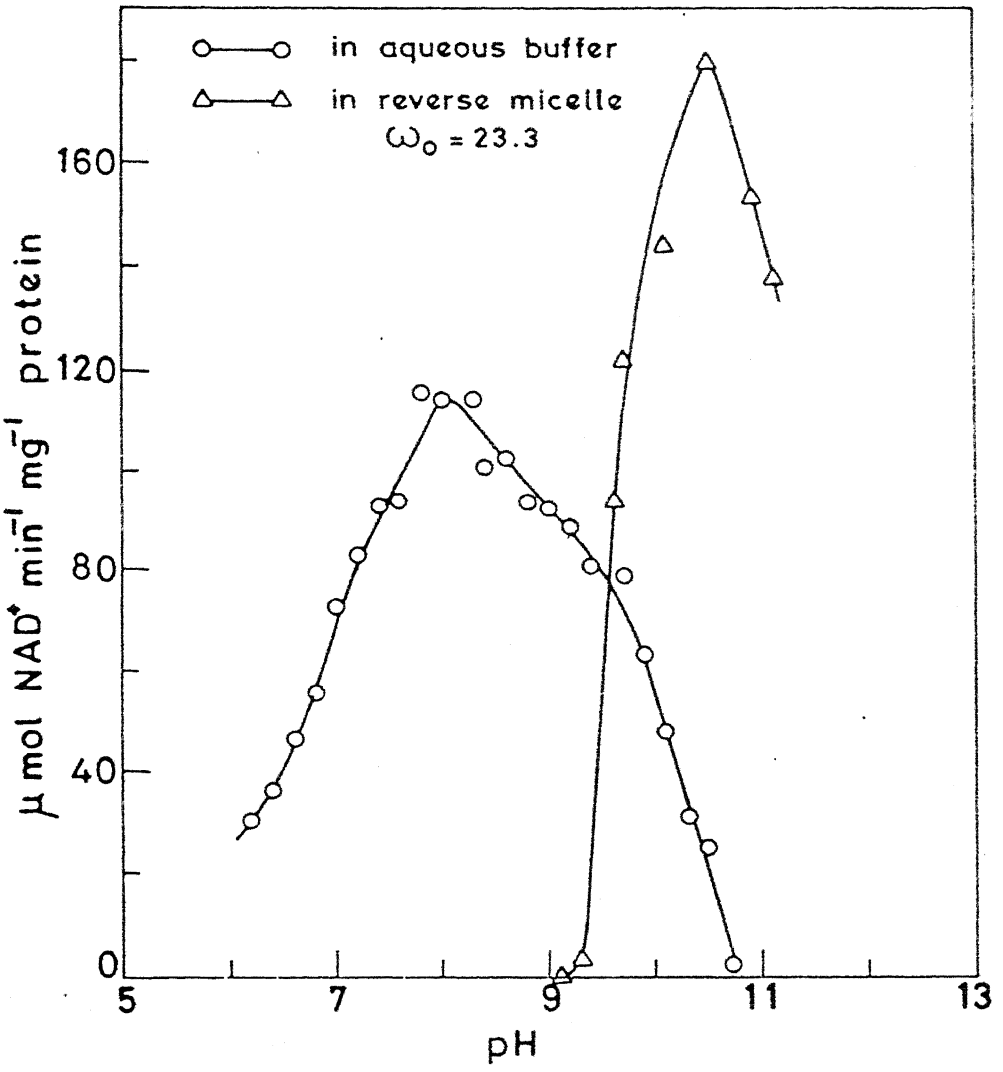

Fig.III.3 Comparison of ADH(Y) activity in aqueous buffer with reverse micellar solution of AOT-isooctane with change of pH. The concentrations were [AOT] = 100 mM, [NAD+] = 0.5 mM and [C_2H_5OH] = 100 mM. Buffers were 100 mM phosphate (pH = 6-8.0), 10 mM pyrophosphate (pH = 8.3) and 100 mM glycine-KOH (pH = 8.4-11.1).

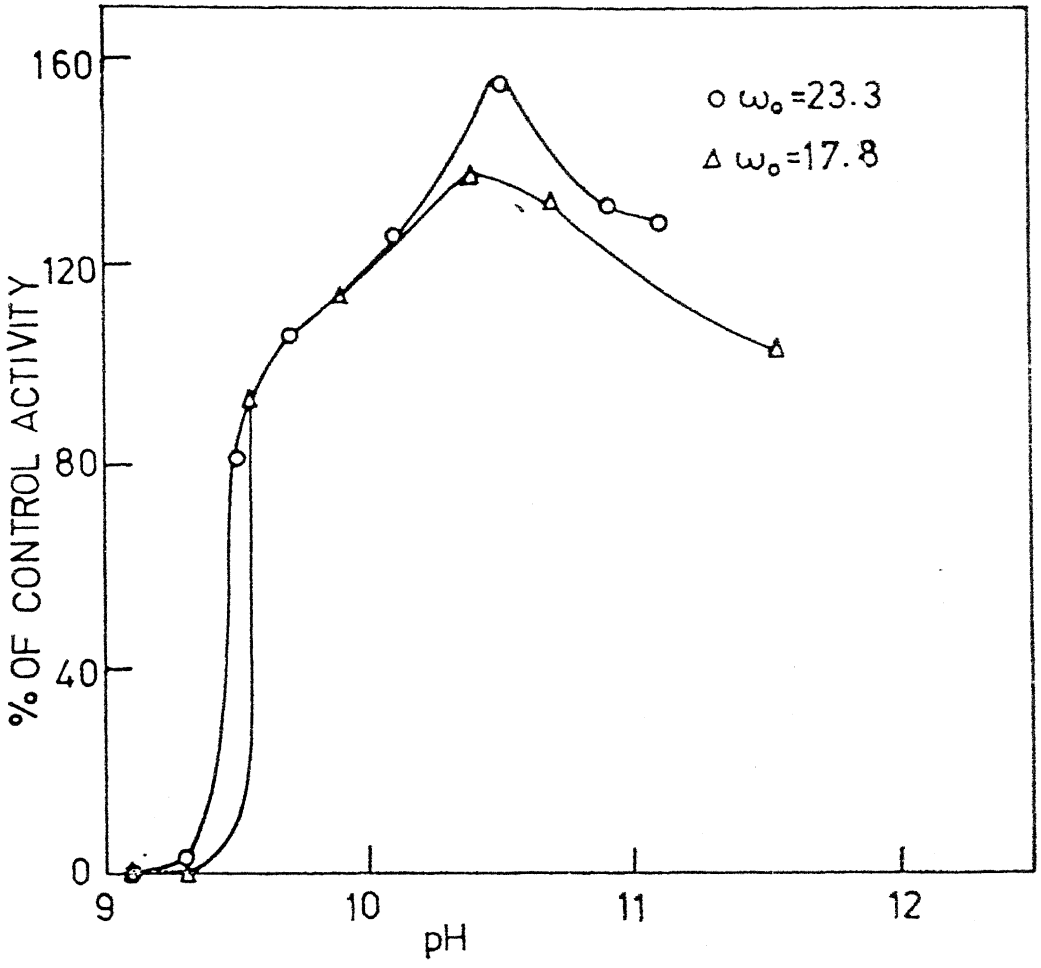

Fig.III.4 Activity profile of ADH(Y) in AOT/isooctane reverse micelles as a function of pH at different water pools. The activity is expressed relative to the activity in aqueous buffer. Other concentrations were same as in Fig. III.3.

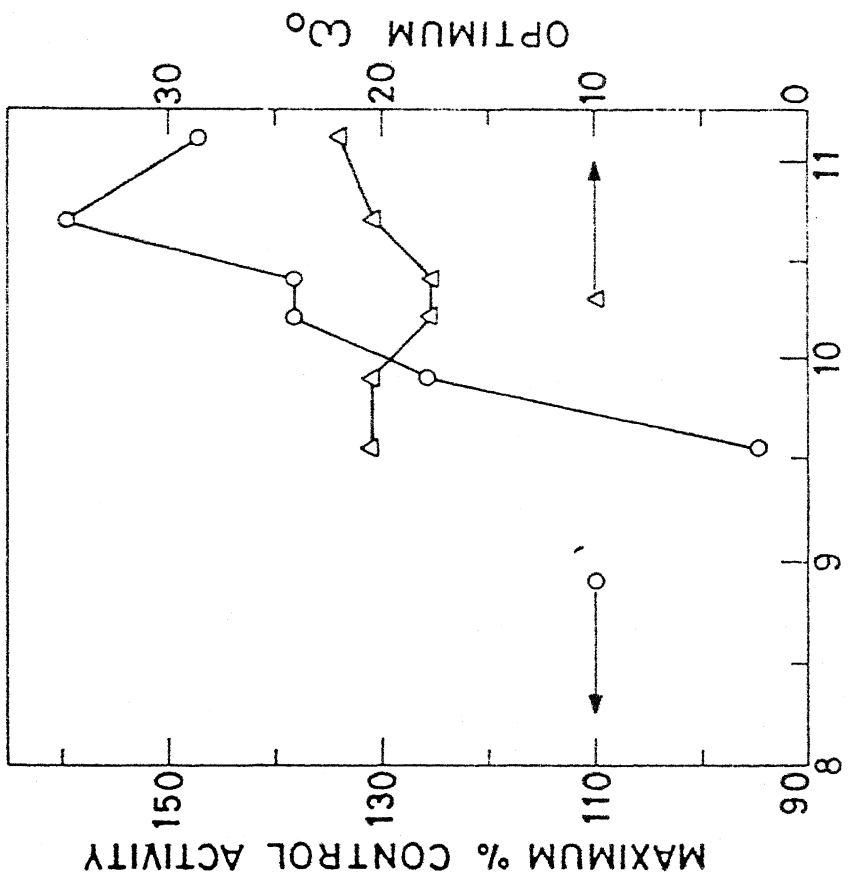

Fig.III.5 Variation of maximum percentage control activity (o—o) and optimum w_o (Δ—Δ) with the pH for AOT(Y) in AOT/isooctane reverse micellar system.

kind of observation where maximum activity in reverse micelles shifted by 2-3 units are known in literature.[24,25] There are reports in which the actual pH of a solution inside the micelle increases by few units.[25] The pK_a values of different amino acids which take active part at the active site may be modified in the new environment of water pool. It may be noted that this enzyme is not active at all below pH 9.1 and again its activity decreases at higher pH. Above pH 9.5, its activity is above 100% i.e. superactive.

Maximum percentage control activity and optimum w_o have been plotted against pH in Fig. III.5. There is sharp change of maximum percentage control activity with pH. Maximum percentage control activity increases upto pH = 10.7 and beyond that it decreases. There is not much variation of $w_{o,opt}$ with pH.

III.3.3.3 Effect of Surfactant Concentration on Enzyme Activity

The influence of Aerosol OT concentration on enzyme activity is significant and it is shown in Fig. III.6 and Fig. III.7. The investigation has been carried out in two different conditions: (a) keeping water pool constant (Fig. III.6) and (b) keeping percentage of water amount invariant (Fig. III.7).

At fixed w_o the effect of increasing surfactant concentration on enzyme activity showed an increase first, reached a maximum and then decreased. The activity showed a maximum at 0.15M surfactant concentration and at this point activity was 172% of the activity in aqueous buffer solution under identical concentrations of NAD^+, ethyl alcohol and enzyme.

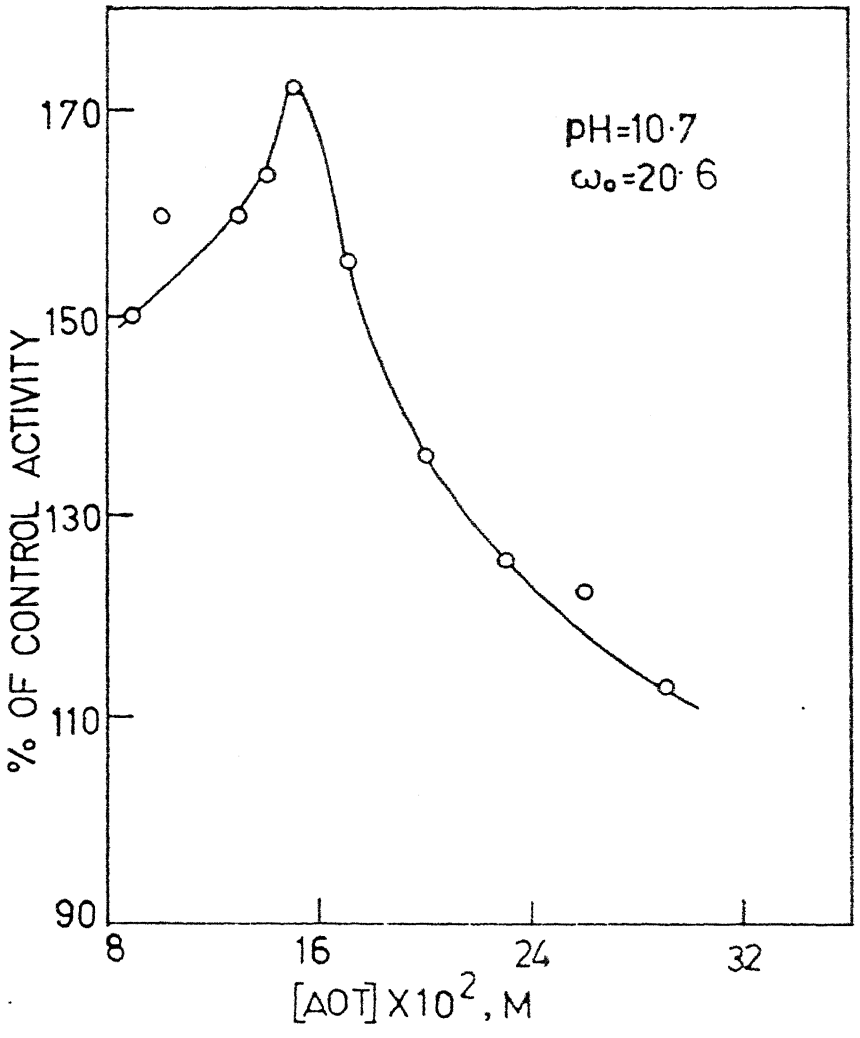

Fig.III.6 Percentage control activity versus surfactant concentration profile at fixed w_o. The concentrations were $[NAD^+]$ = 0.5 mM and $[C_2H_5OH]$ = 100 mM. Buffer used was 100 mM glycine-KOH.

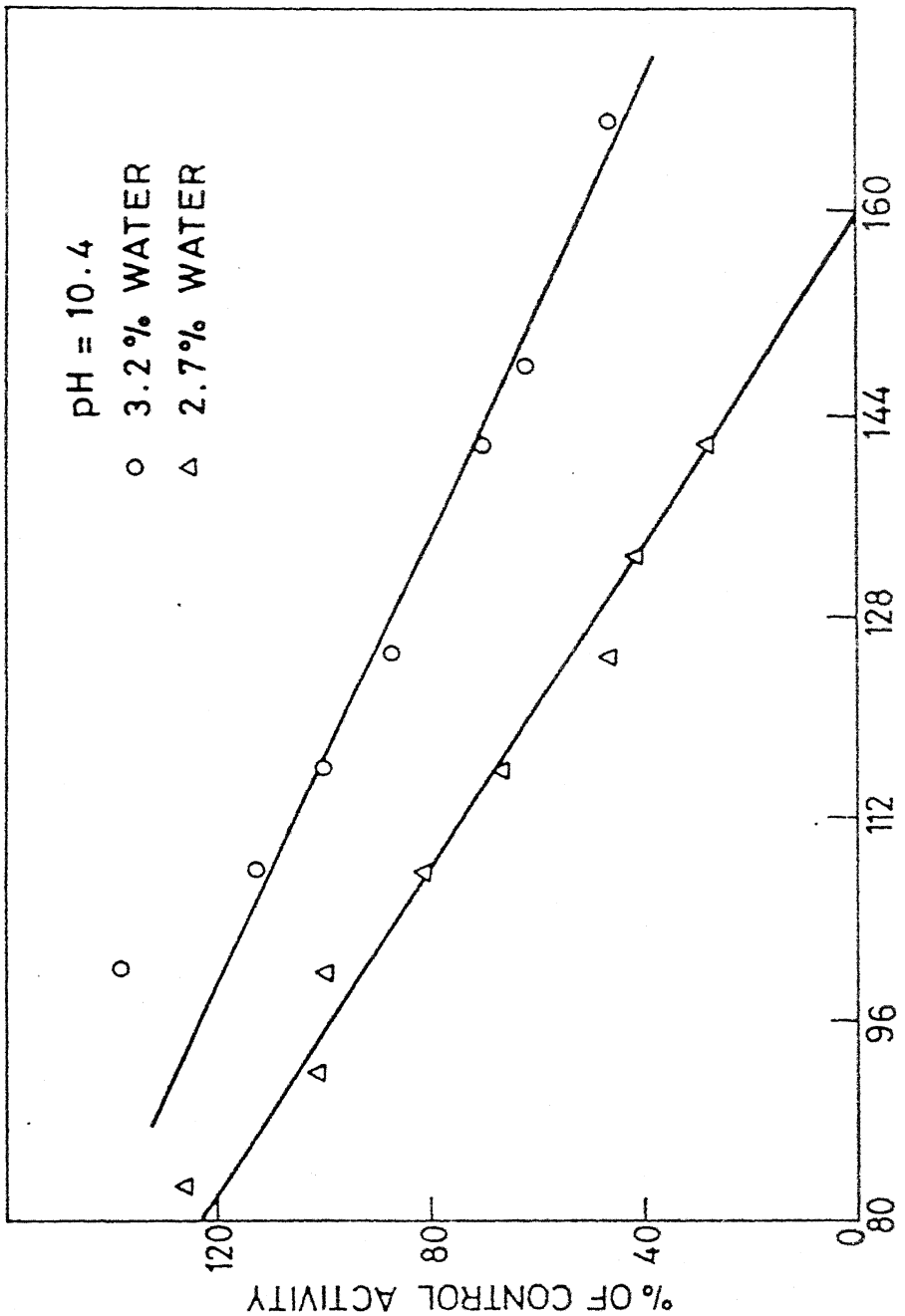

Fig.III.7 Percentage control activity versus surfactant concentration profile keeping percentage of water fixed. Other concentrations were same as in Fig. III.6.

Throughout the AOT concentration range 0.09 M - 0.29 M, its activity was more than its activity in aqueous buffer. At higher concentration of surfactant the activity decreases. It can be explained in the following way. When concentration of surfactant increases the number of micelles which are accommodating the reactants will increase. The mean free path for collision of two micelles decreases and hence the number of collisions per second increases and thus fusion of two micelles enhances. In this process the contents of two micelles get more exposure to solvent which might be the reason for decrease of activity with increasing concentration of surfactant. At low surfactant concentration, the surfactant might not be able to provide the protective compartment from organic solvent. Another reason may be that at low surfactant concentration the micelles are not sufficiently tight. Though the number of micelles are less, but every collision with other micelle makes its hosted material vulnerable to exposure to the bulk organic solvent.

With fixed amount of water the increase in surfactant concentration decreases the enzyme activity linearly. Here increase in surfactant concentration at fixed amount of water means, that the degree of hydration w_o is decreasing continuously. At low water pool enzyme can not accomodate itself in its native conformation inside the reverse micelle and therefore with increasing surfactant concentration at invariant amount of water less and less enzyme activity is observed.

III.3.3.4 Spectral Study of Alcohol Dehydrogenase Reactions

In biochemical studies[26] UV/Vis spectroscopy has been used

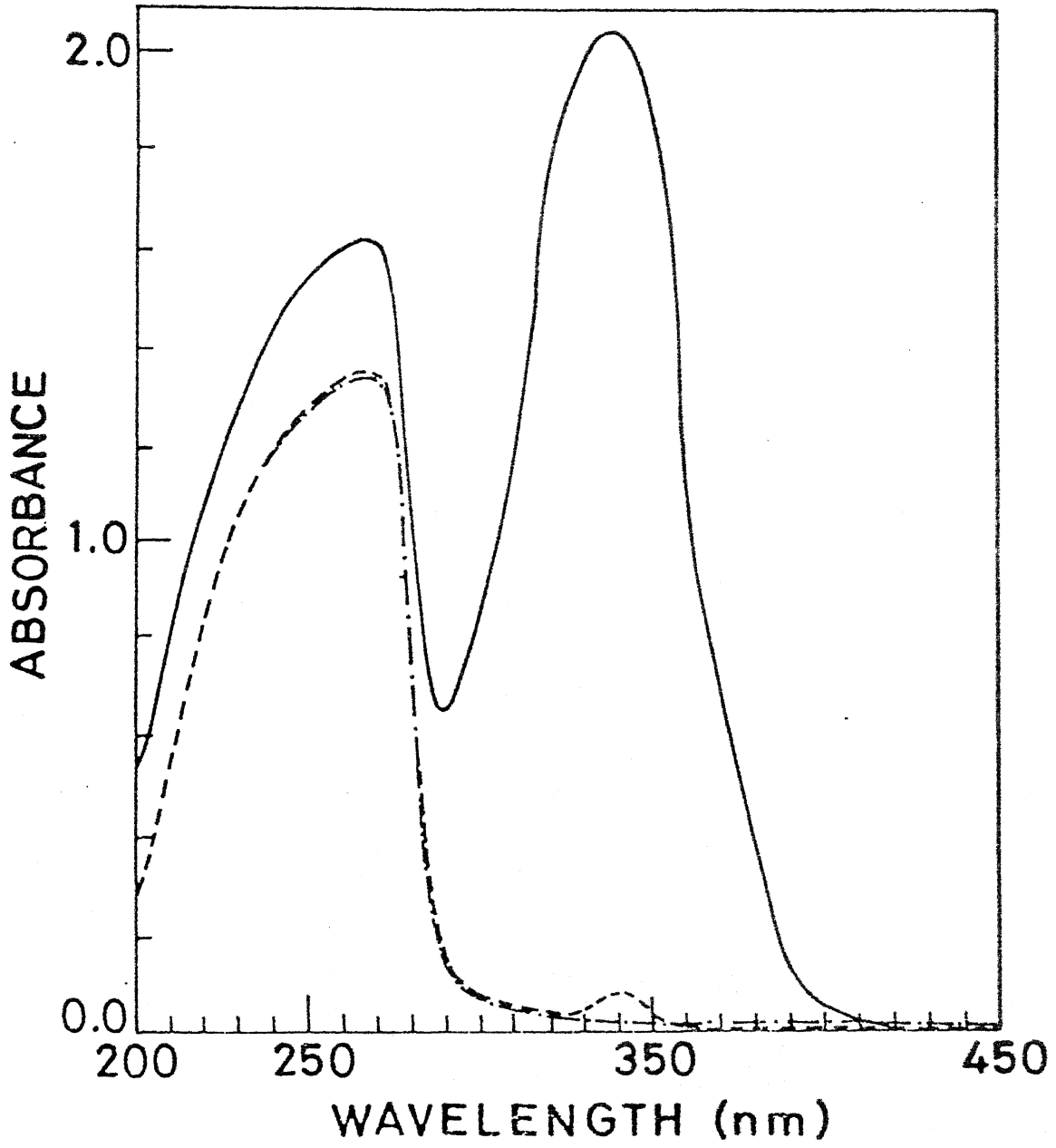

Fig.III.8 Electronic absorption spectra of NAD^+, NADH and the product obtained (after the completion of ADH(Y) catalysed reaction) in aqueous buffer (10 mM pyrophosphate buffer, pH = 8.3).- - - - - NAD^+, ——— NADH and – – – – – product.

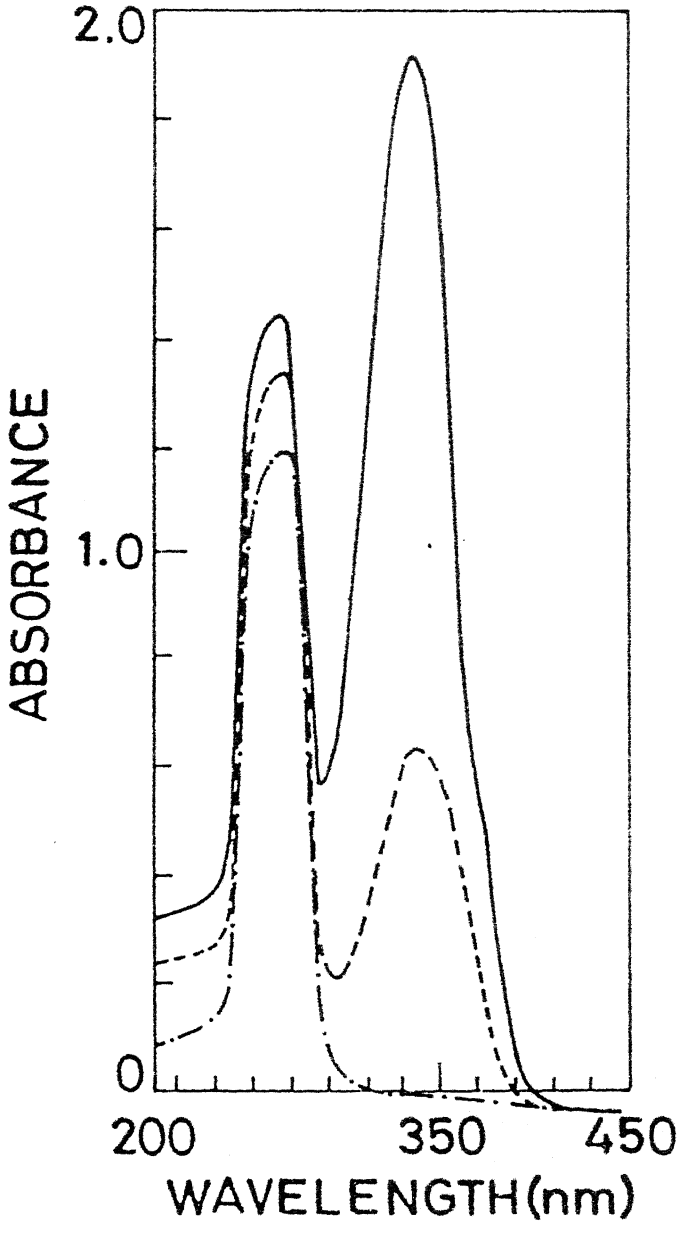

Fig.III.9 Electronic absorption spectra of NAD^+, NADH and the product formed (after the completion of ADH(Y) catalysed reaction) in reverse micellar solution of AOT/isooctane at w_o=20.6, pH = 10.7 (100 mM glycine-KOH). —·—·—·— NAD^+, ———— NADH and – – – – product.

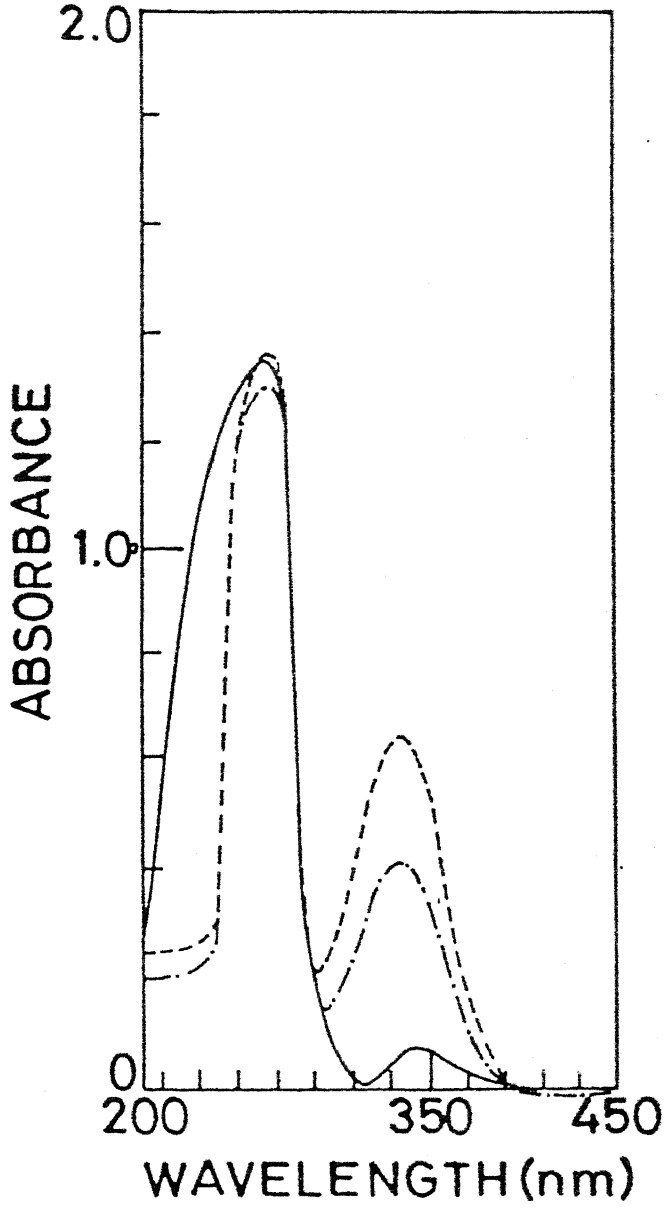

Fig.III.10 Electronic absorption spectra of the product formed (after completion of ADH(Y) catalysed reaction in aqueous buffer (——— pH = 8.3) and in reverse micellar solution at w_o = 20.6, pH = 10.7 (----------) and w_o = 15.00 pH=10.7 (—·—·—·—·—).

extensively for obtaining structural information. The coenzymes NADH and NAD^+ are involved in oxidation-reduction reaction catalysed by alcohol dehydrogenase. The oxidised form of NAD^+ has an absorption maximum at 260 nm due to purine and pyridine rings, but the reduced form show an additional band at 340 nm. Reverse micellar solution of AOT/isooctane forms a clear solution with ADH(Y), NAD^+ and C_2H_5OH and hence absorption spectroscopy can be conveniently used to study the enzyme reaction in this microheterogeneous medium.

To establish the identical nature of the enzyme reaction in aqueous and non-aqueous medium the absorption spectra of the aqueous (buffer medium) and reverse micellar solution, before and after the completion of enzyme reaction were recorded. The absorption spectra of coenzymes NADH, NAD^+ and the corresponding product of enzyme catalysed by ADH(Y) have been recorded in both aqueous (Fig. III.8) and reverse micellar solution (Fig. III.9). Characteristic peak of the product in aqueous solution and in the reverse micelle was same at 340 nm. Fig. III.10 gives the absorption spectra of product formed in the ADH(Y) catalysed reaction in reverse micellar solution at two water pools (w_o = 20.6 and w_o = 15.0) and in aqueous buffer. These spectra at two different water pools are almost identical in themselves but show a small difference from the spectrum in aqueous buffer below 250 nm. The peak in both the media were at 260 nm and 340 nm. Change in the shape of spectrum from aqueous to reverse micellar medium is attributed to the change from polar to apolar environment. The study indicates that same product is formed in both the aqueous and reverse micellar media.

III.3.3.5 Time Dependent Stability Study of Alcohol Dehydrogenase in Reverse Micellar Medium

Time dependent stability study of enzymes in reverse micelles is important because usually enzymes loose their activity slowly in aqueous buffer and there is a possibility that they can be stabilised for longer time in reverse micelles if suitable optimum conditions are sorted out. In Fig. III.11 and Fig. III.12 we have shown the variation of percent residual activity of enzyme as a function of time with different combinations of substrate, coenzyme and water pools at fixed pH. The control was obtained by measuring the activity of enzyme at zero time. A parallel and similar experiment was done by observing the time dependent stability in aqueous medium. Fig. III.11 shows that the enzyme is more stable in aqueous medium than in reverse micelle. In reverse micelle the enzyme almost looses its activity fast whether it is put in reverse micelle alone or with substrates. In aqueous buffer medium the enzyme retains 60% activity when it is incubated with C_2H_5OH but retains 80% activity in presence of NAD^+ for 30 minutes. The enzyme looses its activity completely within 17 hours. From the plot it is obvious that at any time the enzyme retains more residual activity when it is incubated with NAD^+. It becomes obvious that binding of NAD^+ with enzyme protects its active site from deactivation. Similar observation is obtained in reverse micelles too. If we consider the incubation time of 10-15 mins. in reverse micelle, the enzyme looses most of its activity in this period though the residual activity for the case where the enzyme is put with NAD^+ is 85%. So it can be inferred that

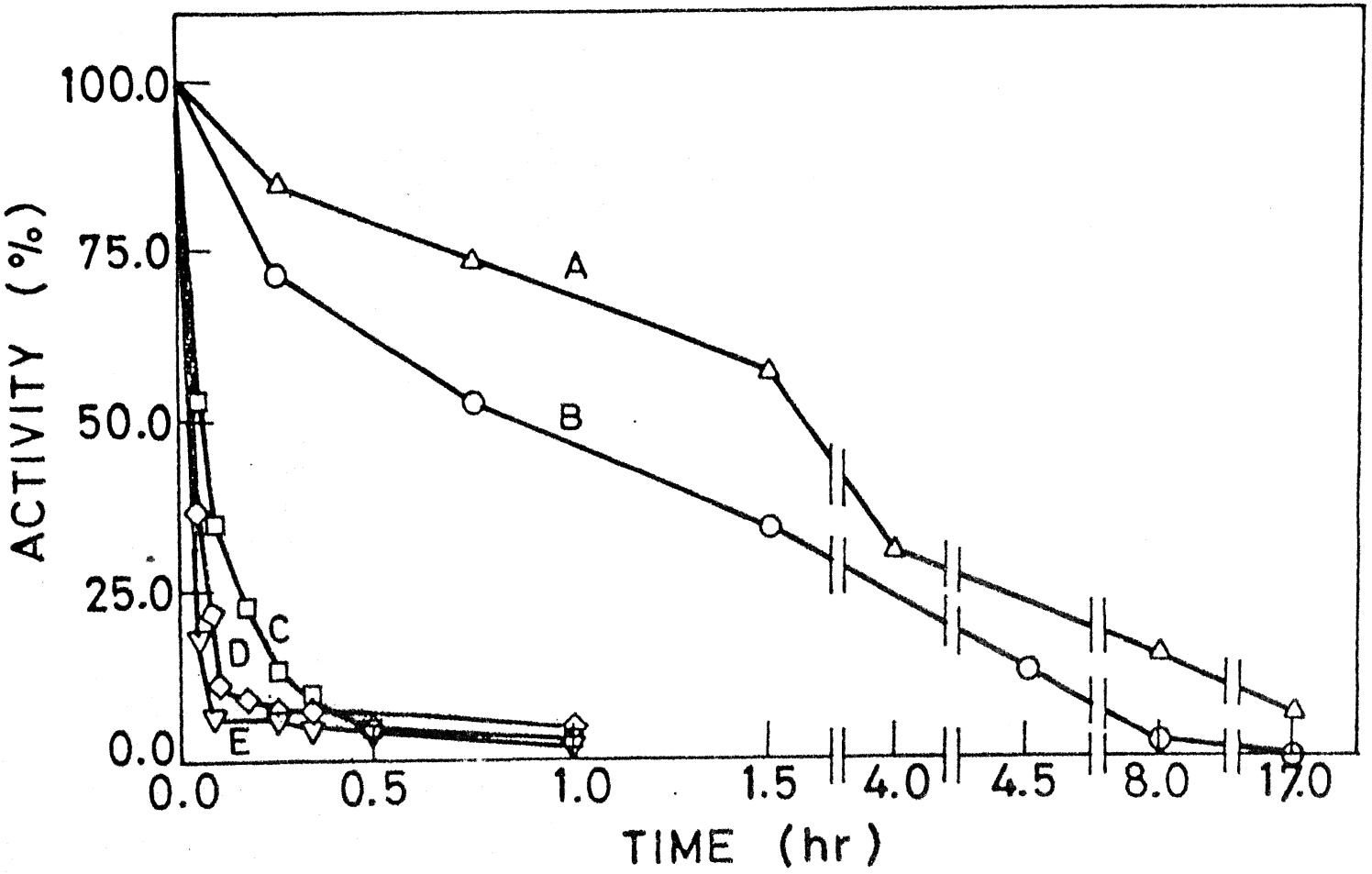

Fig.III.11 Stability of ADH(Y) as a function of time in water (pyrophosphate buffer, 10 mM, pH = 8.3) and in 100 mM AOT/isooctane micellar solution in presence of substrate, coenzyme and w_o = 20.6, pH = 10.70 [A] ADH(Y) + NAD^+; [B] ADH(Y) + C_2H_5OH (both A and B are in aqueous medium); [C] ADH(Y) + NAD^+; [D] ADH(Y); [E] ADH(Y) + C_2H_5OH (all C,D and E are in micellar media).

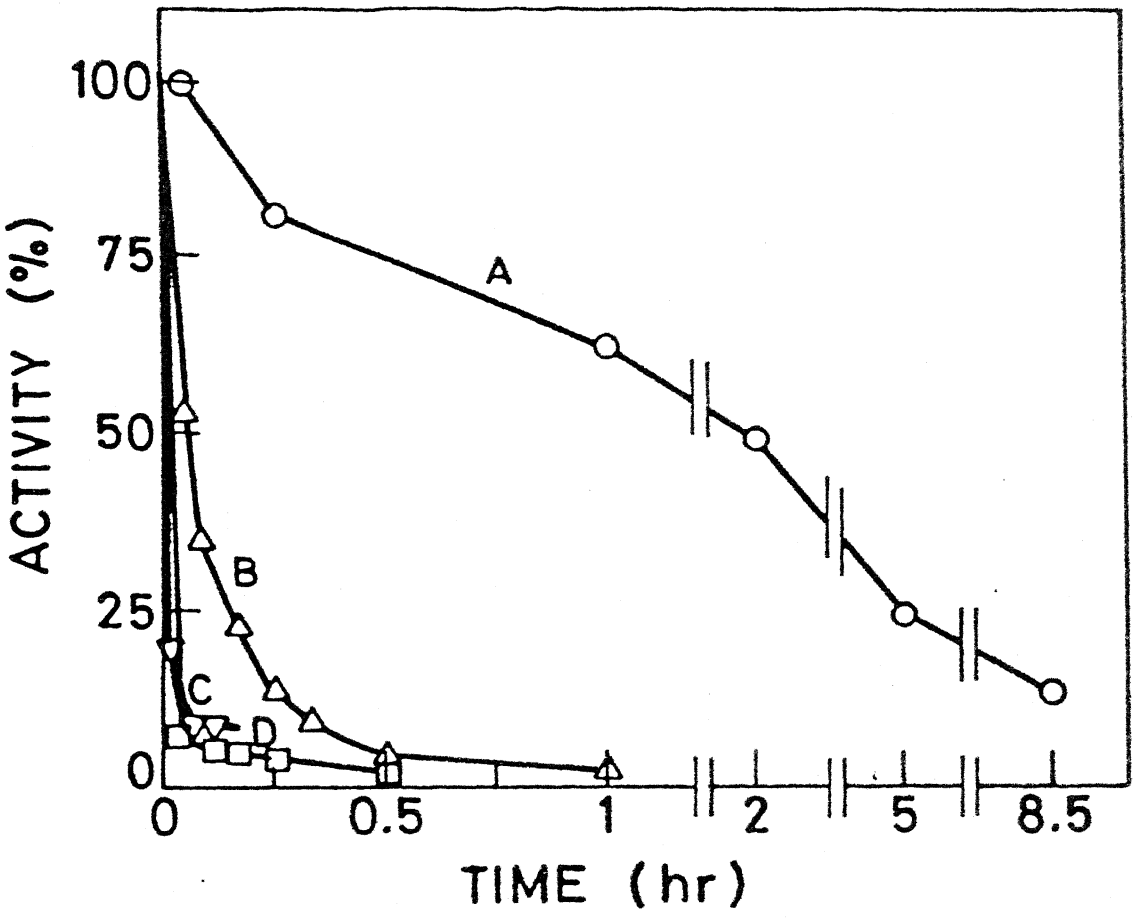

Fig.III.12 Stability of ADH(Y) as a function of time in aqueous and 100 mM. AOT/isooctane reverse micellar solution in presence of NAD^+ at different w_o [A] water, 10 mM pyrophosphate buffer; pH = 8.3; [B] w_o = 20.6; [C] w_o = 17.8; [D] w_o = 23.3. Buffer used was 100 mM glycine-KOH, pH = 10.7.

enzyme is being protected by NAD^+ in reverse micelle. It may be noted that whether it is in aqueous or in reverse micelle the loss of activity at any time of incubation is higher when the enzyme is incubated with ethyl alcohol. It implies that the ability of ethyl alcohol to denature enzyme still exists even when it is a substrate of that enzyme in reverse micelle.

The data of Fig. III.12 which shows the stability of enzyme alone when studied at different water pools in reverse micellar media and in aqueous buffer it is apparent that the enzyme is more stable in aqueous buffer as compared to reverse micellar soloution. In reverse micellar solution within half an hour enzyme looses its activity completely whereas it took almost 8.5 hrs. in buffer. When we compare the residual activity of enzyme at any time of its incubation, its stability is highest when water pool is 20.6 . This study shows that the enzyme is most stable at the water pool at which it shows its maximum activity. This kind of observation was obtained in cases like α - chymotrypsin.[20] Only a couple of enzymes like lipase[27] and α - chymotrypsin[28] have been reported to be stable for few hours in AOT/isooctane reverse micellar solution.

III.3.3.6 Characteristic Kinetic Constants of ADH(Y) Enzyme in Reverse Micellar Solution

Like aqueous buffer solutions, determination of kinetic parameters in hydrocarbon micellar solution is very essential. Michaelis-Menten constant (K_m), V_{max} etc. are the characteristic property of an enzyme in a particular medium.

Enzyme reaction velocity is dependent on enzyme concentration, concentrations of its substrate(s), activators and inhibitors specific for the enzyme, nonspecific effects of compounds (salts and buffers), pH, ionic strength, temperature[29] etc. In reverse micellar solution enzyme activity is also dependent on surfactant type, surfactant concentration, solvent, water pool size etc. Some of these effects have already been discussed. Effect of coenzyme, substrate and enzyme concentrations in reverse micellar media are studied in this section and the kinetic parameters like K_m, V_{max} etc. are determined.

In general it is found that kinetic behavior of enzyme in reverse micellar solution does not change significantly and it obeys Michaelis-Menten kinetics in reverse micelles.

III.3.3.6.1 Effect of Enzyme Concentration on Enzyme Reaction Velocity

In reverse micelles enzyme molecules are made to reside and function in microcaptive environment of water pool. It is essential to check whether enzyme molecules work independently of each other in this microenvironment. Initial velocity of the enzyme reaction has been plotted as a function of enzyme concentration in Fig. III.13. It may be noted that the enzyme reaction velocity is linear upto the enzyme concentration 125 ng/ml. It implies that the molecules of the enzyme function independently in the reverse micellar solution and it is also an

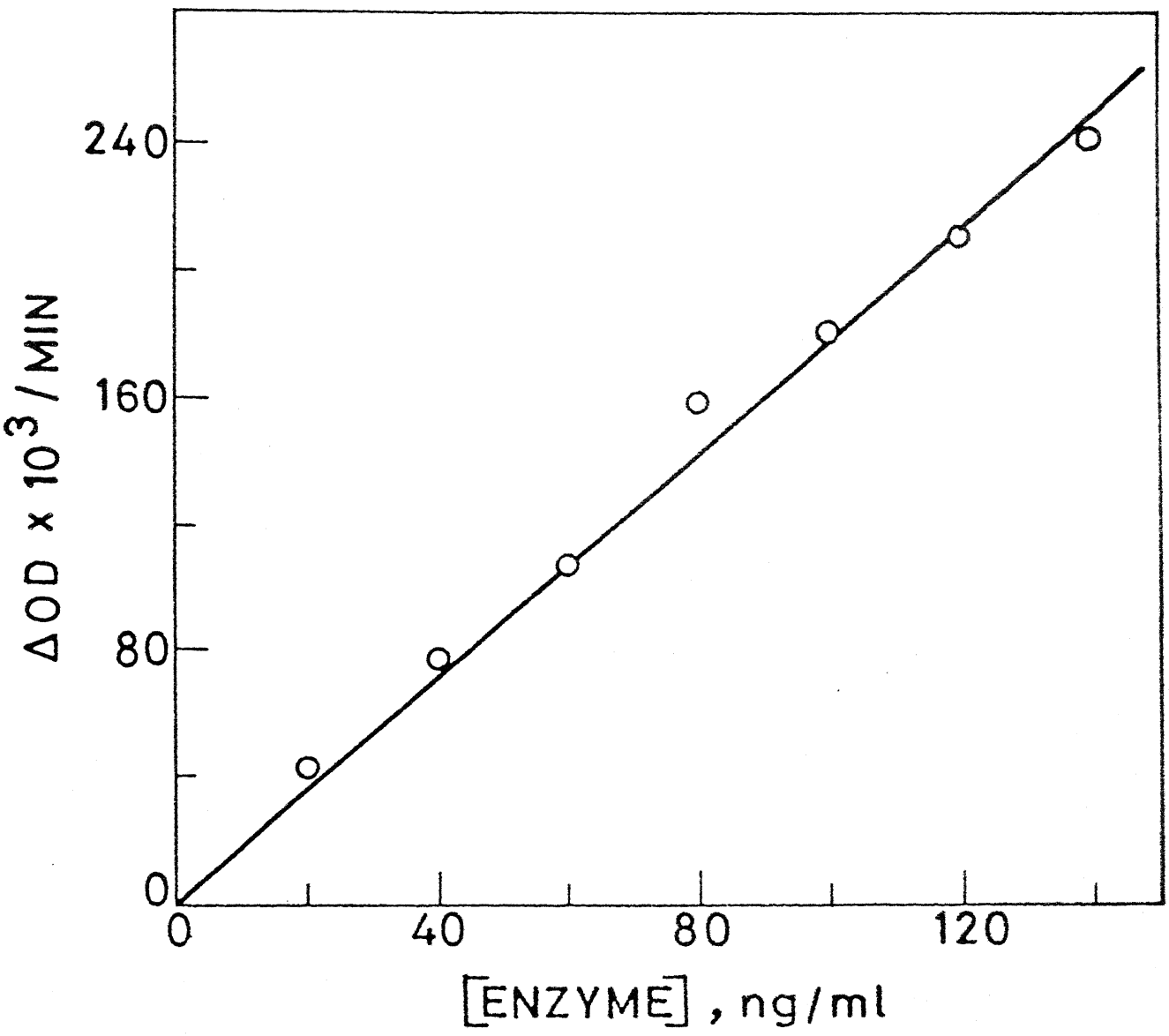

Fig.III.13 Variation of initial velocity of enzyme reaction in reverse micellar solution of AOT in isooctane on ADH(Y) concentration at pH = 10.7, w_o = 20.6, $[C_2H_5OH]$ = 100 mM, $[NAD^+]$ = 0.5 mM. Buffer used was 100 mM glycine-KOH.

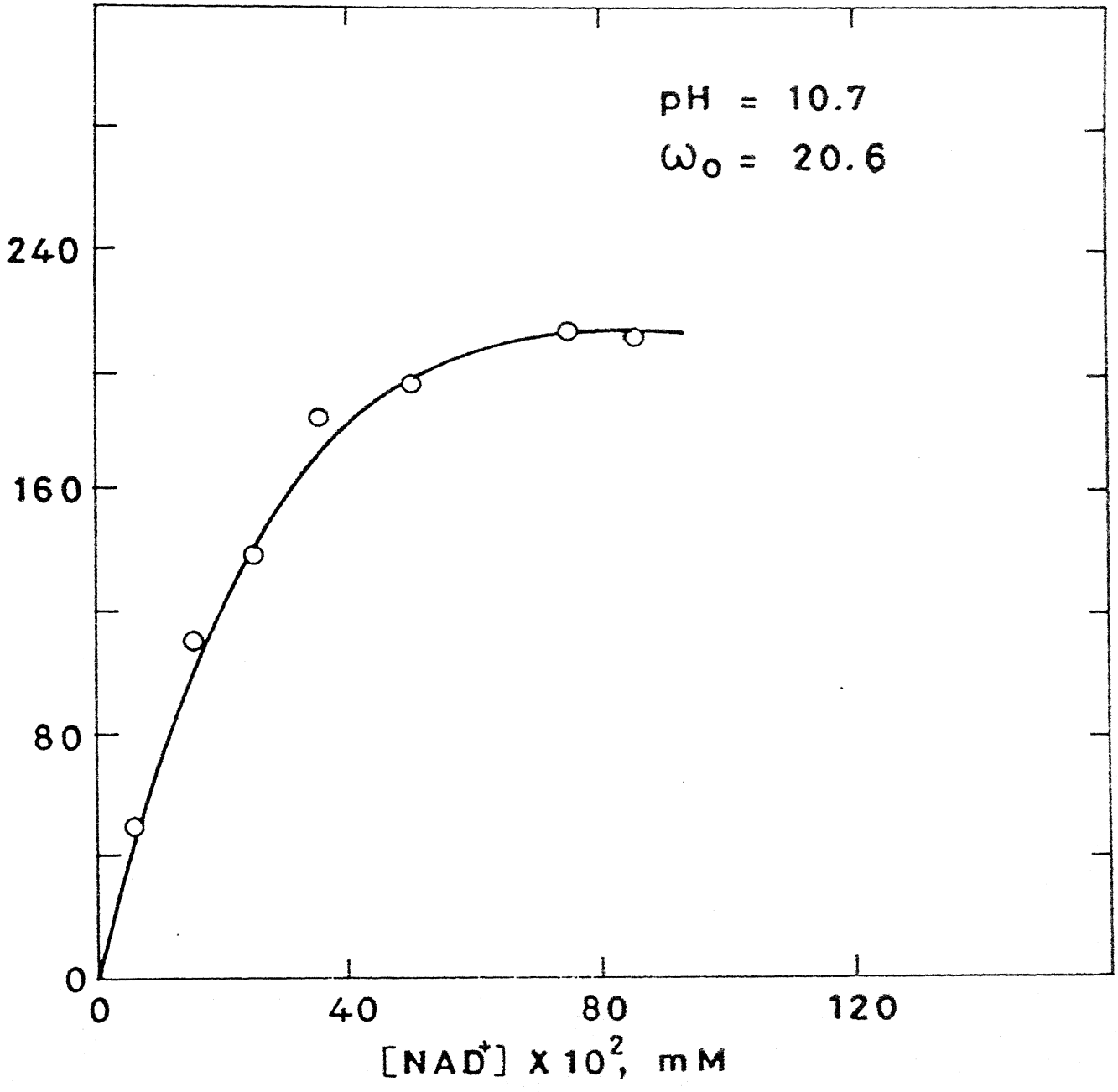

Fig.III.14 Effect of NAD^+ concentrations on ADH(Y) activity in reverse micellar system of AOT/isooctane at w_o=20.6, pH = 10.7. The concentrations were [AOT] = 100 mM, [C_2H_5OH] = 100 mM. The buffer was 100 mM glycine-KOH.

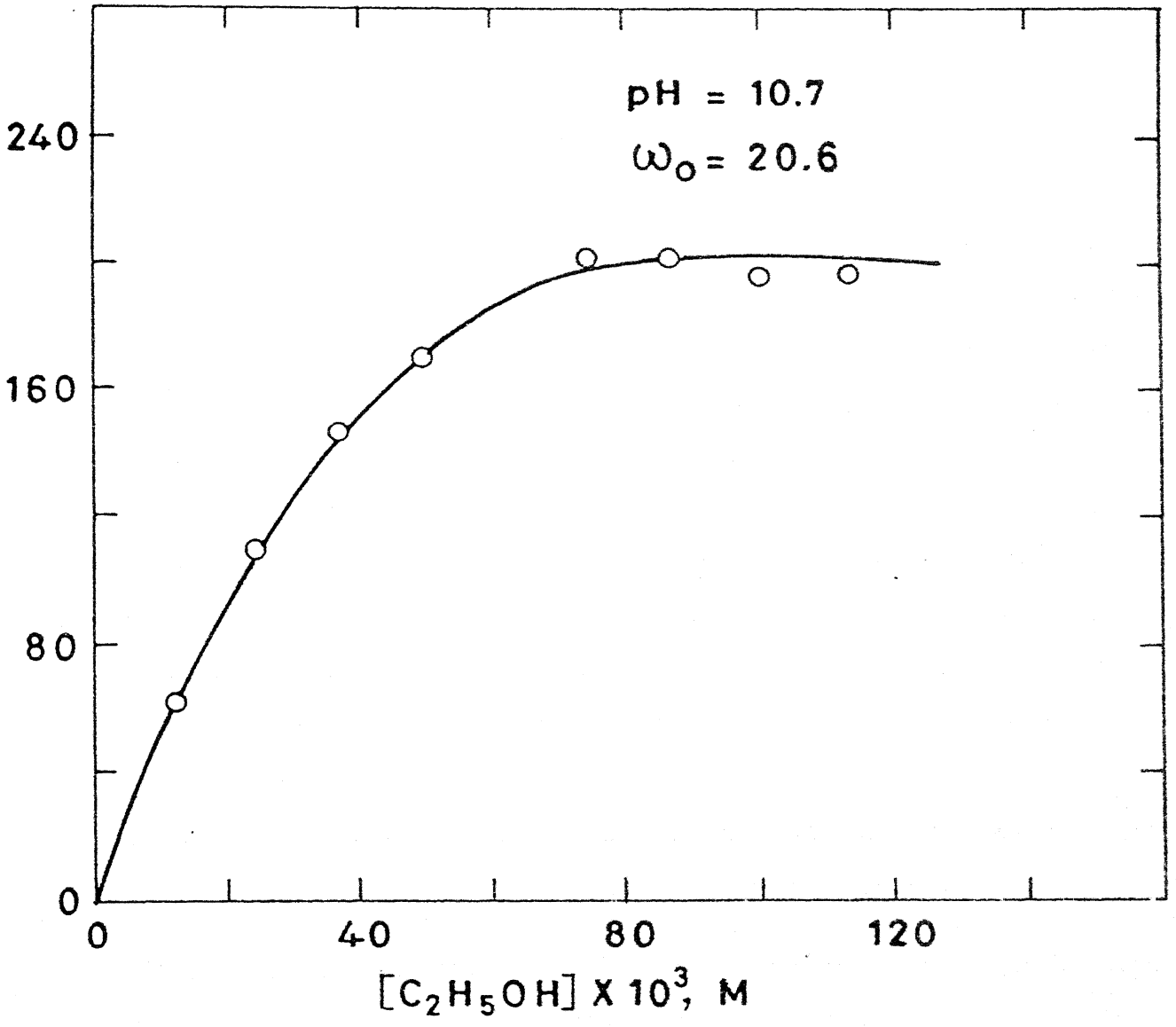

Fig.III.15 Dependence of ADH(Y) specific activity on C_2H_5OH concentrations in 100 mM AOT/isooctane at w_o=20.6 and pH = 10.7. NAD^+ concentration was 0.5 mM and buffer was 100 mM glycine-KOH.

indirect proof that increase in absorbance with time is due to the enzyme reaction which produce NADH.

III.3.3.6.2 Effect of Coenzyme and Substrate Concentration on Reaction Velocity

The activity of yeast alcohol dehydrogenase is dependent on the concentration of coenzyme and substrate. We studied the substrate and coenzyme dependence of enzyme reaction in micellar solution at pH = 10.7 and w_o = 20.6 at which it shows maximum activity. The specific activity of enzyme was determined with the variation of concentration of coenzyme or substrate keeping the concentration of other component fixed. The data on the variation of specific activity versus coenzyme and substrate concentration are given in Fig. III.14 and Fig. III.15. The plots are linear upto 0.3 mM NAD^+ and 60 mM C_2H_5OH. The enzyme reaches the saturation at 0.8 mM NAD^+ and 80 mM C_2H_5OH. It shows that Michaelis-Menten kinetics is followed in both the cases.

III.3.3.6.3 Determination of Kinetic and Binding Parameters of Alcohol Dehydrogenase in Reverse Micelles

Fig. III.16 and Fig. III.17 represents the Lineweaver-Burk plots for the varying concentrations of ethyl alcohol and NAD^+ respectively. The corresponding secondary plots are shown in Fig. III.18 and Fig. III.19. At varying concentration of ethyl alcohol, the fixed concentration of NAD^+ were 0.1 mM, 0.15 mM, 0.20 mM and 0.25 mM. On the other hand when NAD^+ concentration was varied the fixed concentrations of ethyl alcohol were kept at

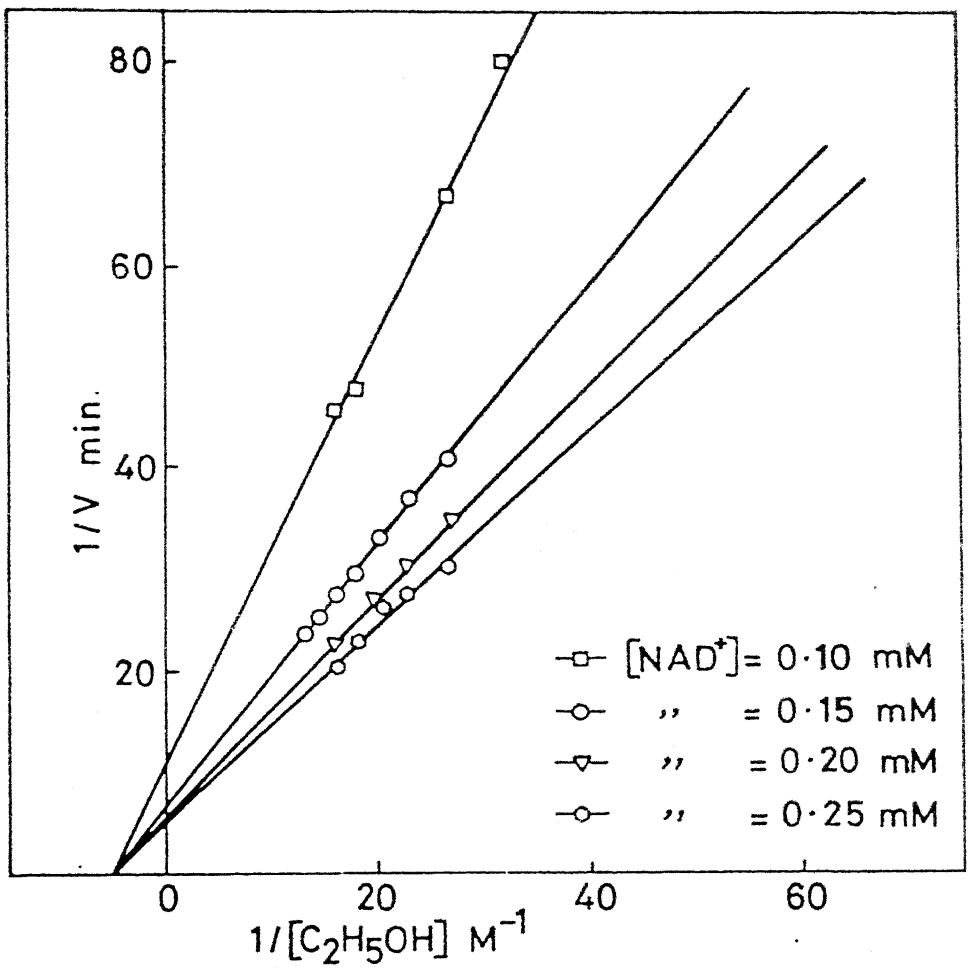

Fig.III.16 Lineweaver-Burk plots for initial ADH(Y) rate with C_2H_5OH concentrations in 100 mM AOT/iso-octane reverse micellar system at different NAD^+ concentrations and w_o = 20.6, pH = 10.7, 100 mM glycine-KOH buffer.

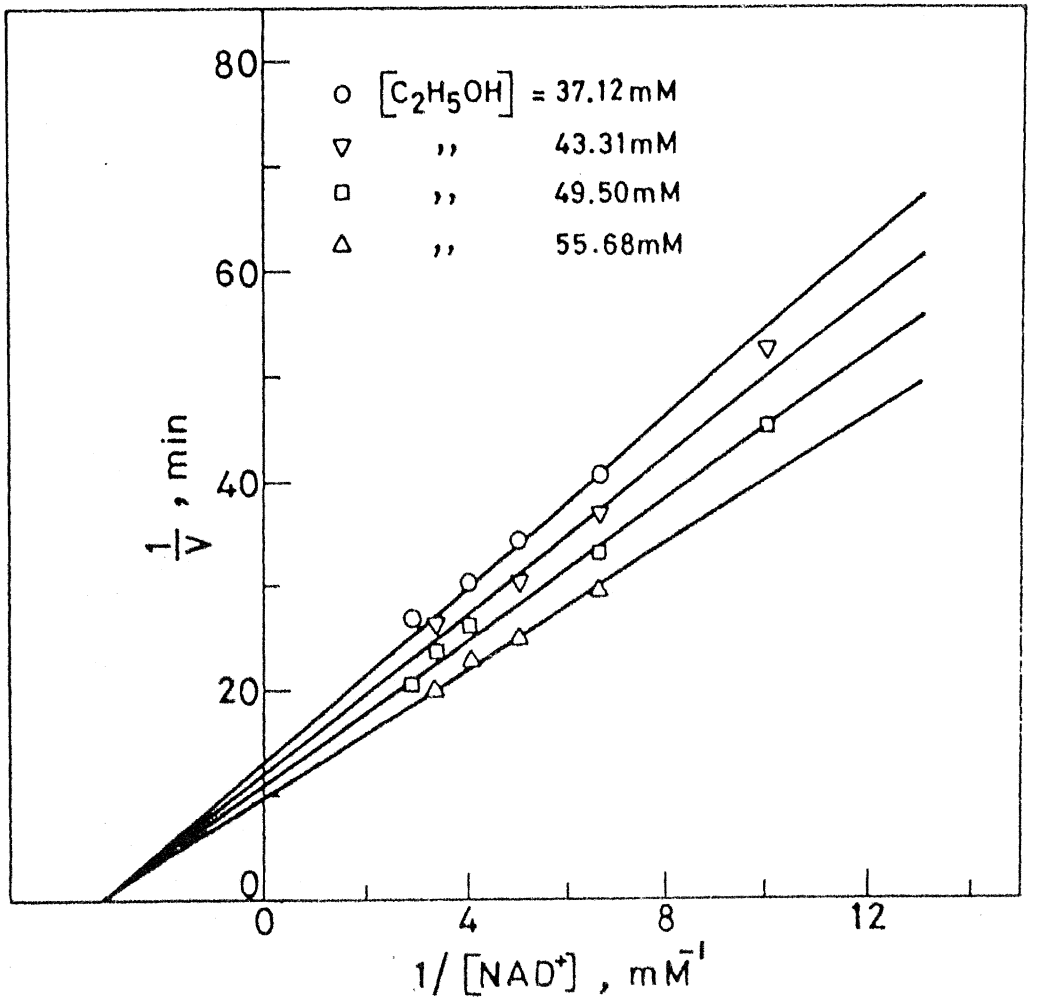

Fig.III.17 Double reciprocal plots for initial ADH(Y) rate with NAD^+ concentrations in 100 mM AOT/isooctane reverse micellar solution at different C_2H_5OH concentrations and w_o = 20.6, pH = 10.7, 100 mM glycine-KOH buffer.

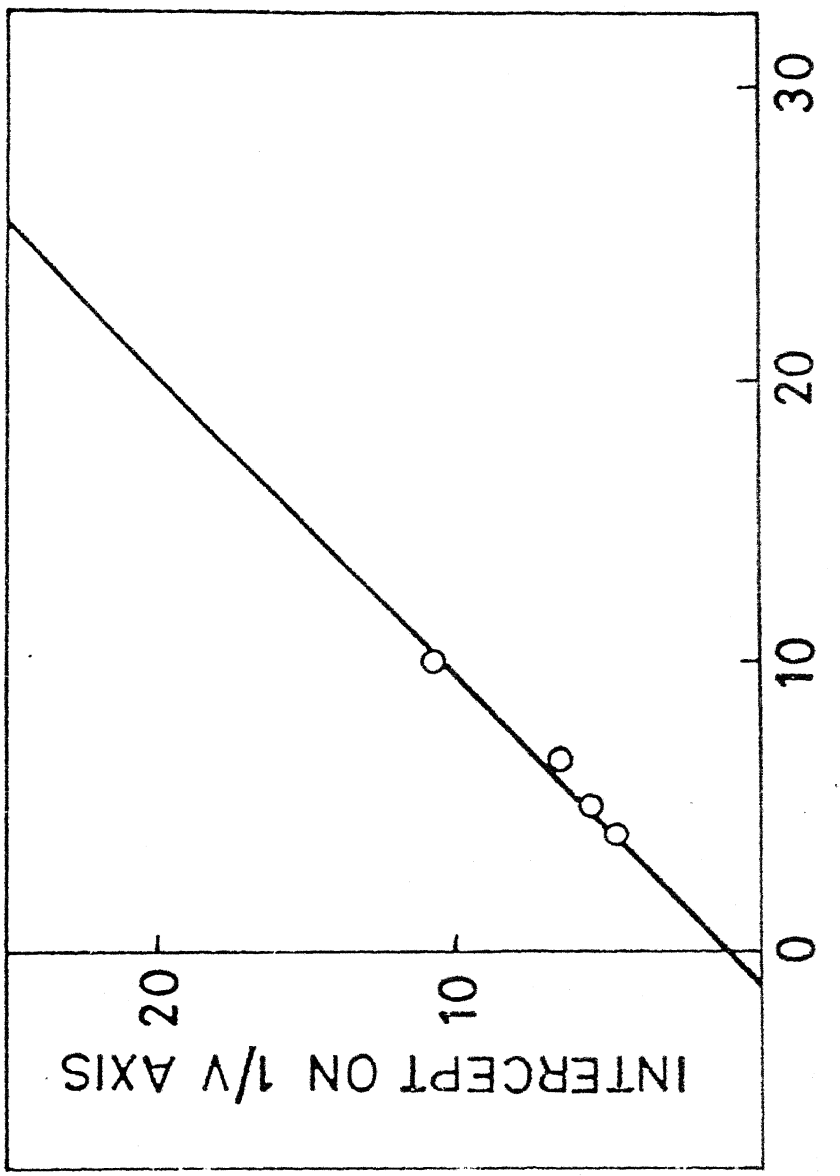

Fig.III.18 Intercept on 1/v axis of Fig.III.16 plotted as a function of inverse NAD^+ concentrations.

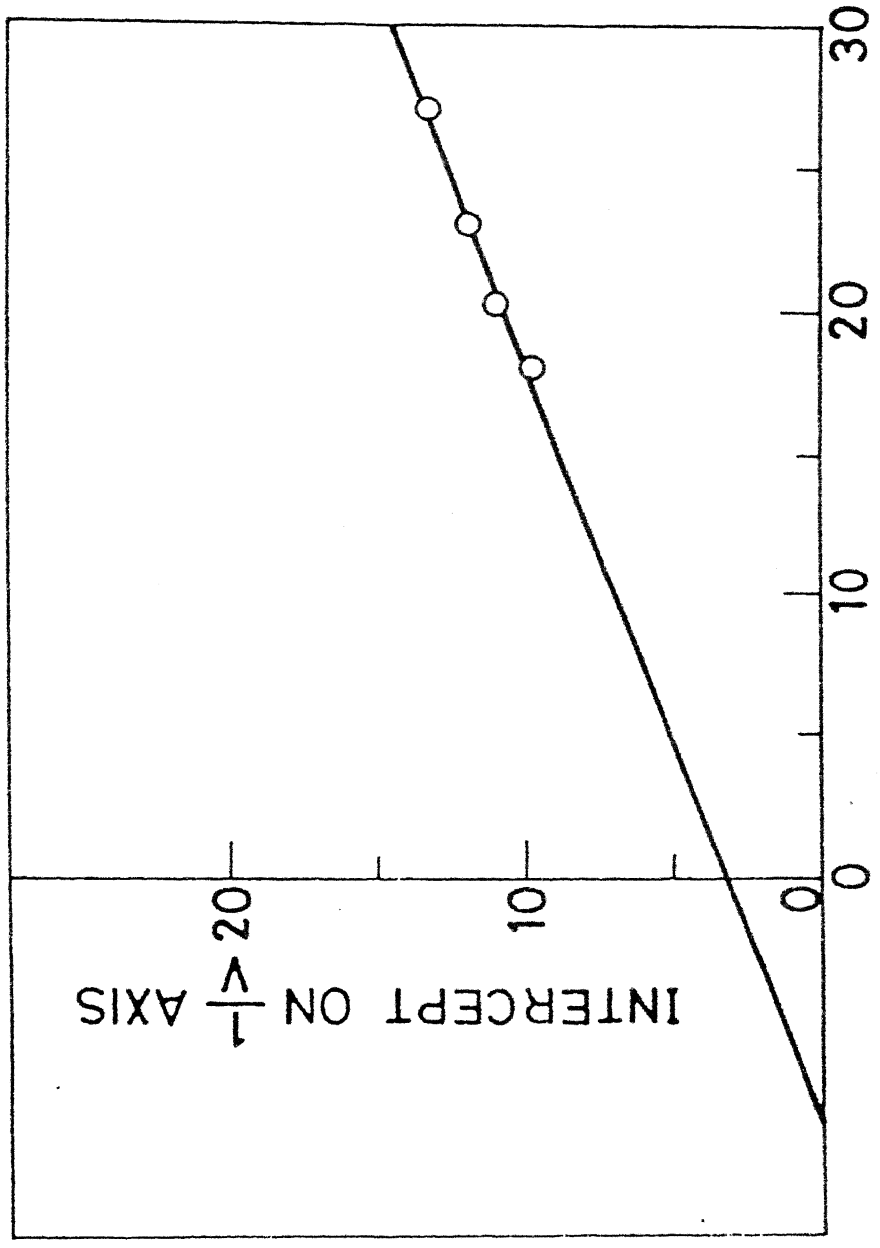

Fig.III.19 Plot of intercept on $1/v$ axis of Fig.III.17 with reciprocal of C_2H_5OH concentrations.

TABLE III.1 Characteristic Constants of Yeast Alcohol Dehydrogenase

System	0.1M AOT in isooctane, w_o=20.6, pH=10.7,100 mM glycine-KOH	Standard deviation	Water pH = 7.15	
$(K_m^{NAD})_{ov}$, M	1.0×10^{-3}	0.139×10^{-3}	7.4×10^{-5}	(30)
$(K_m^{NAD})_{wp}$, M	2.702×10^{-2}	-	-	
$(K_m^{C_2H_5OH})_{ov}$, M	11.764×10^{-2}	1.079×10^{-2}	1.3×10^{-2}	(30)
$(K_m^{C_2H_5OH})_{wp}$, M	3.179×10^{0}	-	-	
$(K_d^{NAD})_{ov}$, M	3.125×10^{-2}	-	-	
$(K_d^{NAD})_{wp}$, M	84.277×10^{-2}	-	-	
V_{max}, μ mol $l^{-1}min^{-1}$ (mg enzyme)$^{-1}$	210.0	-	115.93	

ov, overall

wp, water pool

37.12 mM, 43.31 mM, 49.50 mM and 55.68 mM. The yeast alcohol dehydrogenase in reverse micellar solution follows the kinetic pattern similar to those in aqueous medium.[30] The K_m values calculated from the secondary plots are summarized in Table III.1. It is of interest to note that $(K_m)_{wp}$ is much larger than $(K_m)_{ov}$. $(K_m)_{ov}$ determined for the enzyme is one order of magnitude higher compared to the value obtained in aqueous medium whereas $(K_m)_{wp}$ shows much high value (two order of magnitude) compared to those in water. Since K_m is considered as a good measure of the dissociation constant of the enzyme-substrate complex,[31,32] the large increase of $(K_m)_{ov}$ can be looked as weak binding in between substrates and enzyme. This might be the reason that removal of bound product from enzyme is faster and so the higher activity of yeast alcohol dehydrogenase in reverse micelle. $(K_m)_{ov}$ is more close to K_m in aqueous compared to $(K_m)_{wp}$ with K_m in aqueous. α-Chymotrysin,[20] and lysozyme[25] and horse liver alcohol dehydrogenase[32] in reverse micellar solution have shown similar results. Therefore present study strongly supports the concept that $(K_m)_{ov}$ is a valid K_m in reverse micellar solution in comparison to $(K_m)_{wp}$.

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CHAPTER IV

BEHAVIOR AND KINETIC STUDY OF ALCOHOL DEHYDROGENASE IN CATIONIC REVERSE MICELLAR SOLUTION

IV.1 INTRODUCTION

Alcohol dehydrogenase (EC 1.1.1.1) is an important enzyme in biological systems. Alcohol dehydrogenases from different sources have been extensively studied[1-7] and there is a good potential of exploting this enzyme in organic synthesis and other technological purposes. To extend the use of enzymes in organic solvent for synthesizing improved product attempts have been made to solubilize enzymes in apolar media containing reverse micelles.[8-19] It has been observed that stabilization and solubilization of enzymes[16-18] in reverse micellar water pool is very much dependent on the nature of surfactant. The charge on the surfactant head group highly influences the property of interphase of the water pool. The water pool entrapped in reverse micelle resembles polar pockets in enzymes and provides unique microenvironments for substrate solubilization and interactions. It has been shown[20] that water pools are formed around the polar head groups of surfactant aggregates. The property of water pool wherein enzymes are solubilised depends on

the charge of surfactant head groups. Interactions of water with surfactant head groups in reverse micelles of cationic and anionic surfactant showed different characteristics. It was found that water is bound tighter in cationic reverse micelle than in anionic reverse micelle.[21] Properties of surfactant entrapped water pools are intimately related to the type of surfactant used in the hydrocarbon solvent. For example, effective polarities of water pools in Aerosol OT (an anionic surfactant) reverse micelles varied as a function of added water concentration in the range that corresponded to that between the polarity of methanol and water.[22] Whereas polarities of solubilized water in dodecylammonium propionate (a cationic surfactant):cyclohexane varied between the polarities of pyridine and water[20]). Another important characteristics is that cationic surfactants like cetyltrimethylammonium bromide (CTAB) are in general less prone to denature proteins.[16]

Considering all the above points we found it desirable to study the behavior and kinetic properties of alcohol dehydrogenase in reverse micellar solution of cationic surfactant CTAB in isootane-$CHCl_3$ (1:1, v/v).

IV.2 EXPERIMENTAL SECTION

Materials

Source and purity of the isooctane used in the present study was same as mentioned in the earlier chapter. Cetyltrimethyl-ammonium bromide (CTAB) extrapure grade reagent was obtained from SISCO Research Lab, India. It was further purified following the

reported method[23] and dried over P_2O_5 in an evacuated desiccator for several hours just prior to use. Other chemicals used in the present study have been mentioned in chapter III. Yeast alcohol dehydrogenase, coenzyme NAD^+ and substrate C_2H_5OH were obtained as described in chapter III.

IV.2.1 Methods

The reverse micellar solution containing enzyme, coenzyme and substrate was optically transparent. The procedure followed and instrument used for the present study were the same as mentioned in chapter III. For spectra in reverse micelles, reference taken was the same reverse micellar solution containing identical amount of buffer as in experimental solution.

IV.2.2 Preparation of Enzyme and Substrate Reverse Micellar Solution

Injection method[24] has been found to be suitable for solubilization of the enzyme in reverse micelle of CTAB in isooctane-$CHCl_3$ (1:1, v/v). To 0.9 ml of 0.11 M CTAB solution in isooctane-$CHCl_3$ (1:1, v/v) 2-10 λ of 100 mM NAD^+ solution in Tris-HCl buffer (100 mM) and 3-12 λ of 12.375 M ethanol in glycine-KOH buffer (100 mM) were injected with microsyringes. Additional amount of buffer and solvent were injected to maintain the desired water content and surfactant concentration. The resulting mixture was shaken vigorously on a vortex mixer until the formation of homogeneous (optically transparent) solution.[19,25] The reverse micellar solution becomes optically

transparent by vortexing for 5-10 sec. The reaction was started by adding 3-7 λ of concentrated enzyme solution in pyrophosphate buffer (pH = 8.3, 10 mM), vortexed and then transferred to spectrophotometer cuvette for recording the change in absorbance. Enzyme activity was calculated as described earlier(chapter III).

IV.3.1 Solubilization of the Enzyme in Reverse Micelles of Cationic Surfactant

Alcohol dehydrogenase from yeast gets solubilized in CTAB/isooctane-$CHCl_3$ (1:1, v/v) reverse micellar solution with a small amount of buffer. The solubility of this enzyme in this system is dependent on w_o, pH and temperature etc. Concentration of enzyme and salt in which enzyme is stored regulates solubility of enzyme in reverse micelles. When diluted stock solution of enzyme is injected into the reverse micelle and lightly vortexed it forms an optically transparent solution. This indicates that the enzyme is solubilized according to water-shell model. According to this model enzyme molecule resides inside the water pool of reverse micelle and surrounded by layers of water molecules. The enzyme molecules thus avoid the considerable influence of surfactant molecules and organic solvent. The enzyme containing micelles, coenzyme and substrate containing micelles, the micelles containing enzyme-substrate complex and those unfilled ones are considered to be in rapid equilibrium i.e. they exchange the material hosted inside.

Concentration of solubilized enzyme or substrate in the reverse micelle has been presented as the overall concentration

throughout unless stated otherwise. Yeast alcohol dehydrogenase ADH(Y) is a relatively big enzyme of M.W. ~ 151,000 dalton having 4 sub-units. The concentrated commercial stock solution of this enzyme is diluted 1500 times to entrap it in water core of reverse micelle. The concentration of aqueous stock solution of ADH(Y) at this dilution was 0.02 mg/ml. Glycine-KOH buffer of 100 mM was found to be suitable to assay this enzyme in reverse micelle.

IV.3.2 Dependence of Enzyme Activity on Degree of Hydration

Study of enzyme activity in reverse micellar solution as a function of degree of hydration (w_o) is important to optimise the conditions for maximum activity in a particular micellar solution.[26,27] The variation of specific activity of ADH(Y) with respect to the degree of hydration at pH 10.0 in reverse micellar media is shown in Fig. IV.1. It may be noted that the enzyme activity increases with increasing values of w_o, reaches a maximum and then decreases, the lowest activity being at the w_o values less than 10. Maximum activity of the enzyme is 39.09% of control in aqueous buffer at w_o = 15.0 and pH = 10.0 compared to its maximum activity in aqueous buffer. Fig. IV.2 gives the data on the variation of percentage control activity of the enzyme as a function of w_o at different pH values. The plots show sharp dependence of activity on w_o in the range 12-20.

The bell-shaped nature of activity w_o profile in reverse micelle is general and has been observed for other enzymes viz. α-chymotrypsin,[28] β-hydroxybutyrate dehydrogenase,[29] isocitrate

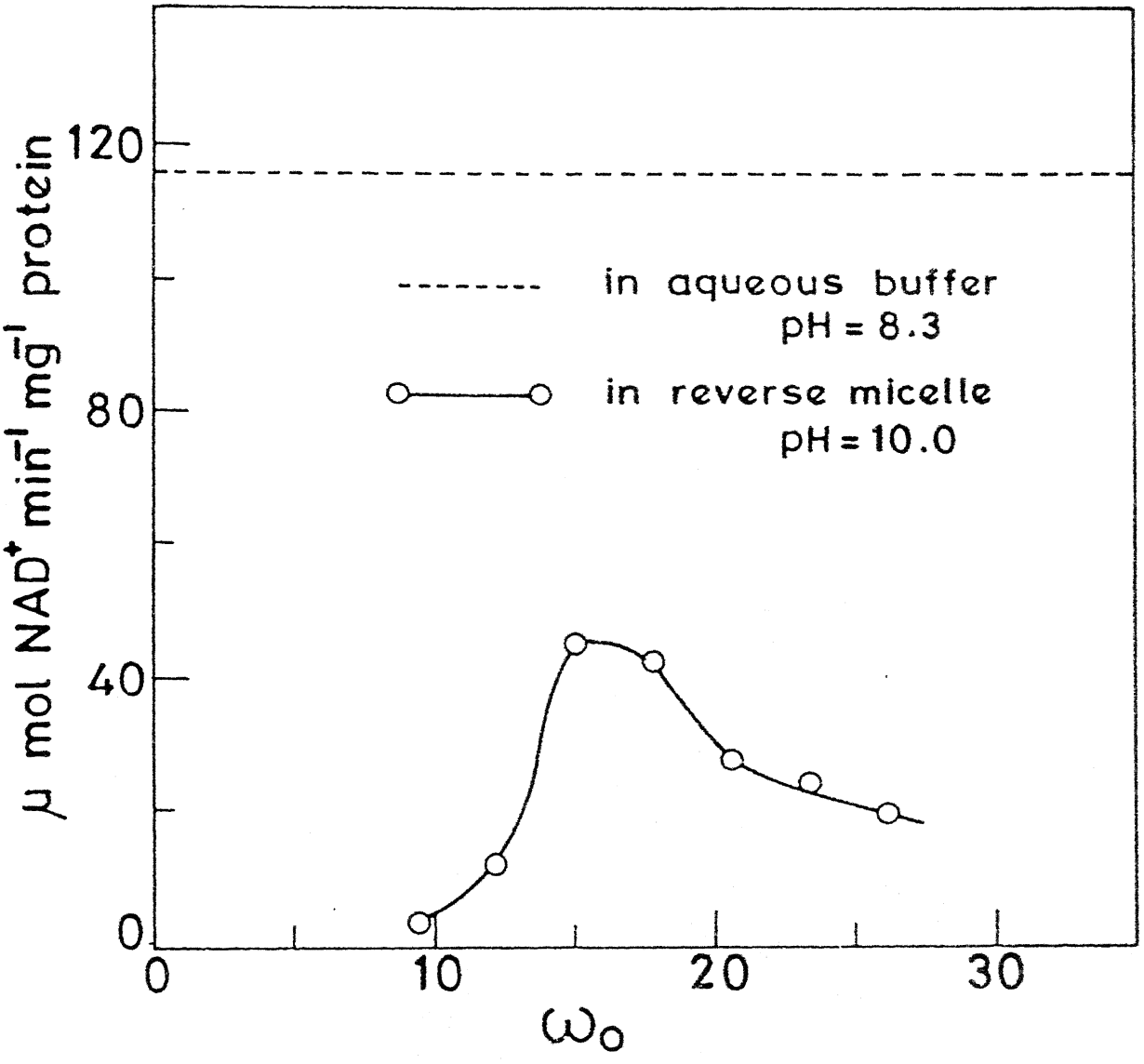
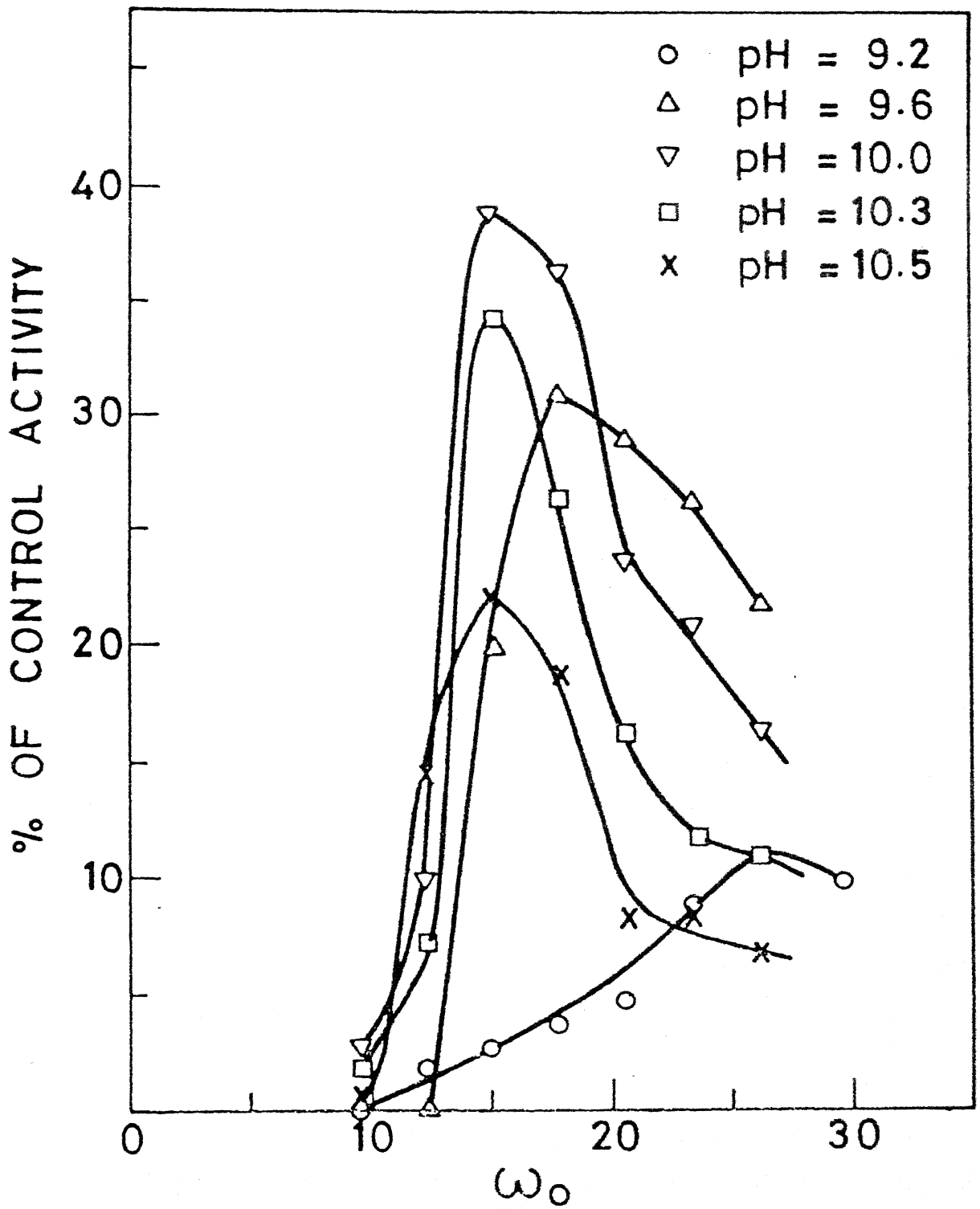


Fig.IV.1 Specific activity of ADH(Y) in CTAB/isooctane-chloroform (1:1,v/v) reverse micelles as a function of w_o. The concentrations were [CTAB] = 100 mM; [C_2H_5OH] = 100 mM and [NAD^+] = 0.5 mM. Buffer was 100 mM Glycine-KOH. [ADH(Y)] = 0.08 μg/ml.



.IV.2 Percentage control activity of ADH(Y) in CTAB/isooctane-chloroform (1:1,v/v) reverse micelles plotted as a function of w_o at different pH values. Buffer was 100 mM glycine-KOH. Surfactant, substrate, coenzyme concentrations were the same as in fig.IV.1.

dehydrogenase,[29] alkaline phosphatase,[30] lipase,[31] lysozyme,[32] trypsin,[33] pyrophosphatase,[17] peroxidase,[34] lactate dehydrogenase,[35] glutathione reductase,[36] dihydrofolate reductase[37] etc. It is possible that the relatively big enzyme ADH(Y) can not assume native conformation within the water pool without being affected by the surfactant and/or solvent when water pool size is less than 10. The decrease in catalytic activity beyond $w_{o,opt}$ may be due to the decrease in interfacial tensions. However the optimum activity is only 40%. Showing thereby that the CTAB system forming reverse micelles with positively charged interior is not conducive for enzyme activity. This is in sharp contrast to reverse micellar solution of anionic surfactant (AOT/ isooctane) where ADH(Y) showed super activity (chapter III).

IV.3.3 Effect of pH on Enzyme Activity

The activity and kinetic parameters of an enzyme are very much sensitive to pH of the buffer solution. As in the earlier case the term pH has been used in place of pH stock (pH of the buffer solution injected into the reverse micellar solution). This pH stock may or may not be the actual pH inside the core of reverse micelles.

Variation of enzyme activity with pH at a particular water pool (e.g. w_o = 15.0 where the enzyme shows maximum activity with water pool variation) has been compared with that of aqueous buffer in Fig. IV.3. Like aqueous medium the dependence of specific activity on reverse micelle is a bell-shaped curve. The striking feature is that whereas in aqueous buffer the enzyme

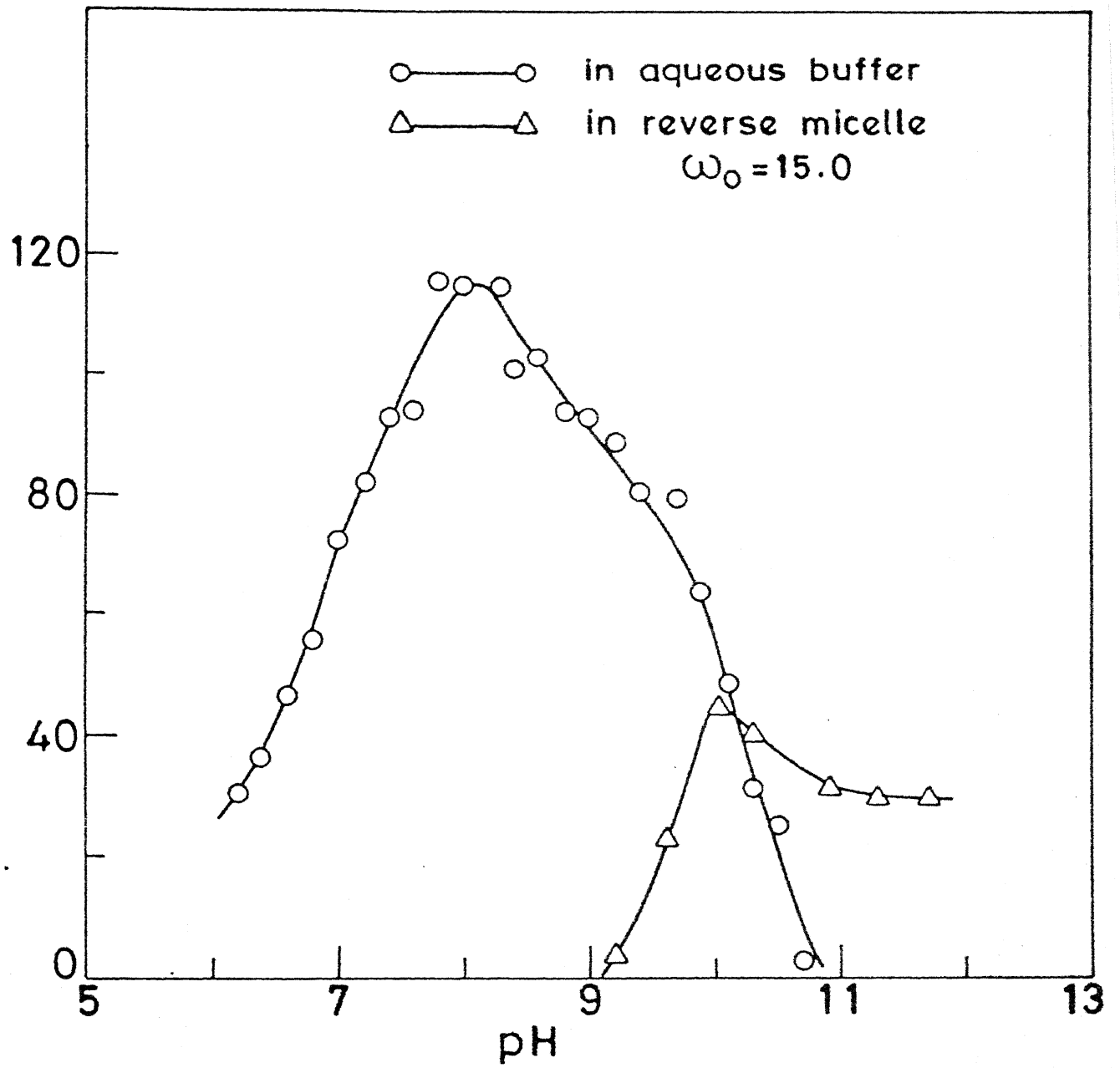
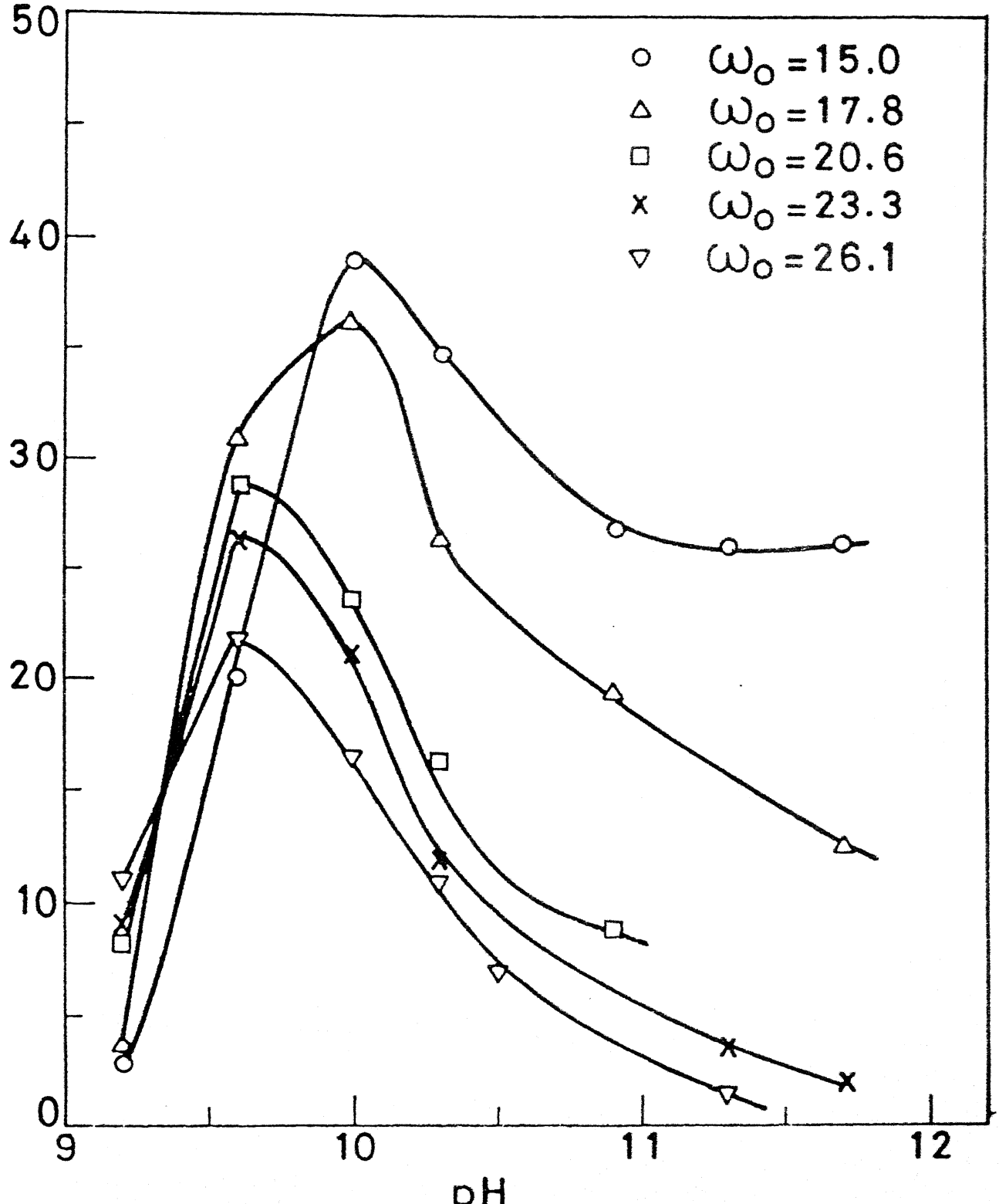
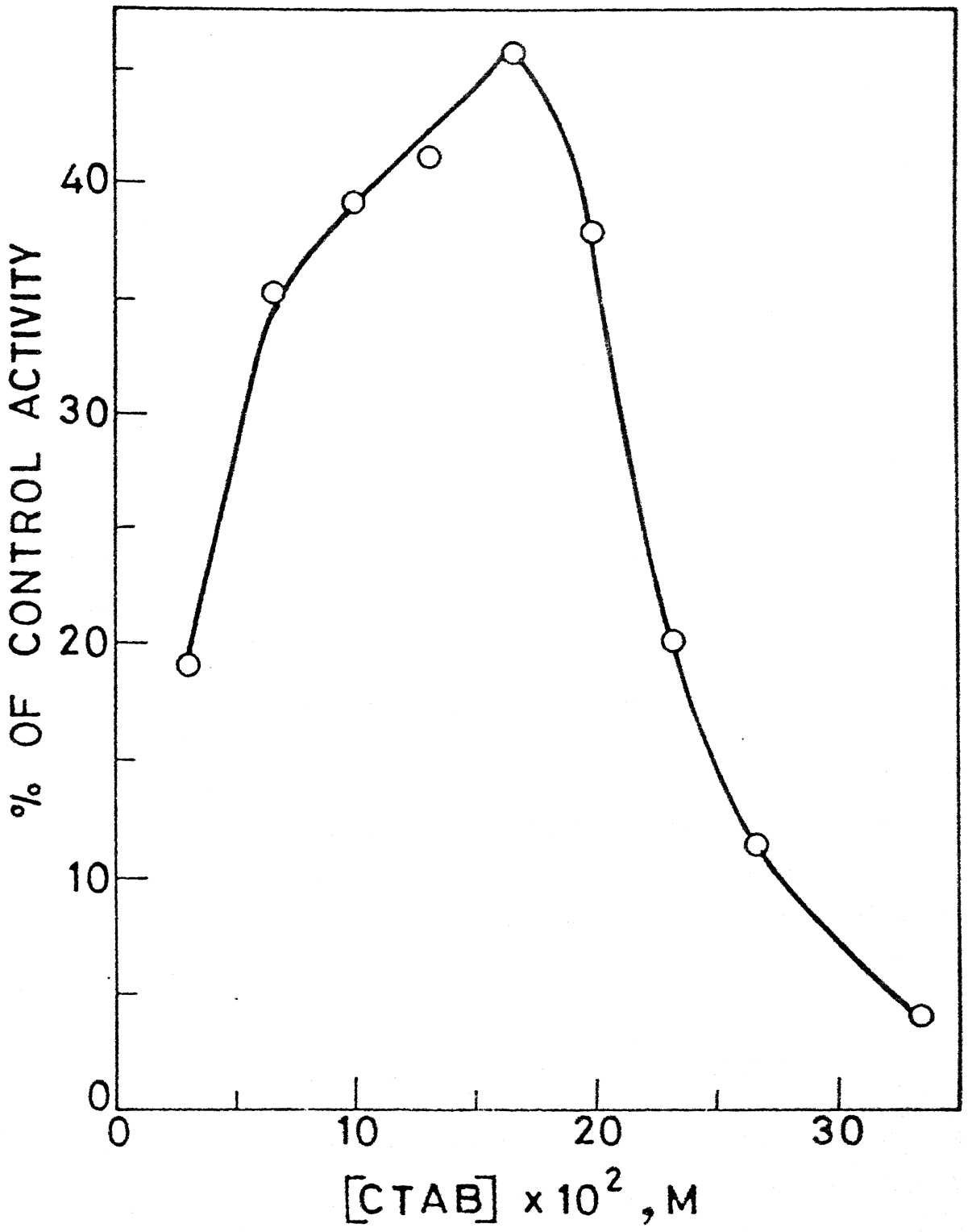


Fig.IV.3 Comparison of ADH(Y) activity in aqueoues buffer and reverse micellar solution of CTAB in isooctane-chloroform (1:1,v/v) with change of pH. The concentrations were [CTAB] = 100 mM; [C_2H_5OH] = 100 mM and [NAD^+] = 0.5 mM. Buffers were 100 mM phosphate (pH = 6-8.0), 10 mM pyrophosphate (pH = 8.3) and 100 mM glycine-KOH (pH = 8.4-11.1).



IV.4 Activity profile of ADH(Y) in CTAB/isooctane-chloroform (1:1,v/v) reverse micelles as a function of pH at different water pools. The activity is expressed relative to the activity in aqueous buffer. Other concentrations were same as in Fig. IV.3.



.IV.5 Effect of surfactant concentration on percentage control activity of ADH(Y) in CTAB/isooctane-chloroform (1:1,v/v) reverse micellar system at pH = 10.0, w_o = 15.0. The concentrations were $[NAD^+]$ = 0.5 mM; $[C_2H_5OH]$ = 0.1 M. Buffer used was 100 mM glycine-KOH.

activity sharply falls beyond pH 9 and becomes zero when pH is around 11, in reverse micelle the enzyme exhibits its activity beyond pH 9 and after pH 11 its activity more or less remains same. Thus in aqueous buffer its maximum activity is around pH 8 whereas in reverse micelle it is at pH 10. This may be due to change in pK_a of certain amino acid residues at the active site of enzyme. Fig. IV.4 shows the effect of pH on ADH(Y) activity in reverse micelles at different w_o. The profile of percentage control activity as a function of pH is similar to other enzymes like lipase,[31] alkaline phosphatase,[38] α-chymotrypsin,[28] lysozyme,[32] trypsin,[33] malate dehydrogenase,[19] glutathione reductase[36] etc.

IV.3.4 Influence of Surfactant Concentration of Enzyme Activity

Formation of reverse micelle and their properties are highly dependent on surfactant concentration. Fig. IV.5 shows the change of enzyme activity with surfactant concentration at w_o = 15.0 and pH 10. The enzyme shows a maximum activity of 47% at 0.167 M CTAB.

IV.3.5 Spectral Study of ADH(Y) Catalysed Reaction

To establish the nature of the enzyme reaction in water (buffer) and reverse micellar solution absorption spectra have been recorded before and after the completion of enzyme reaction. Fig. IV.6 presents the absorption spectra of NAD^+, NADH and the corresponding product of enzyme reaction catalysed by ADH(Y). The spectra in aqueous buffer medium was reported earlier in

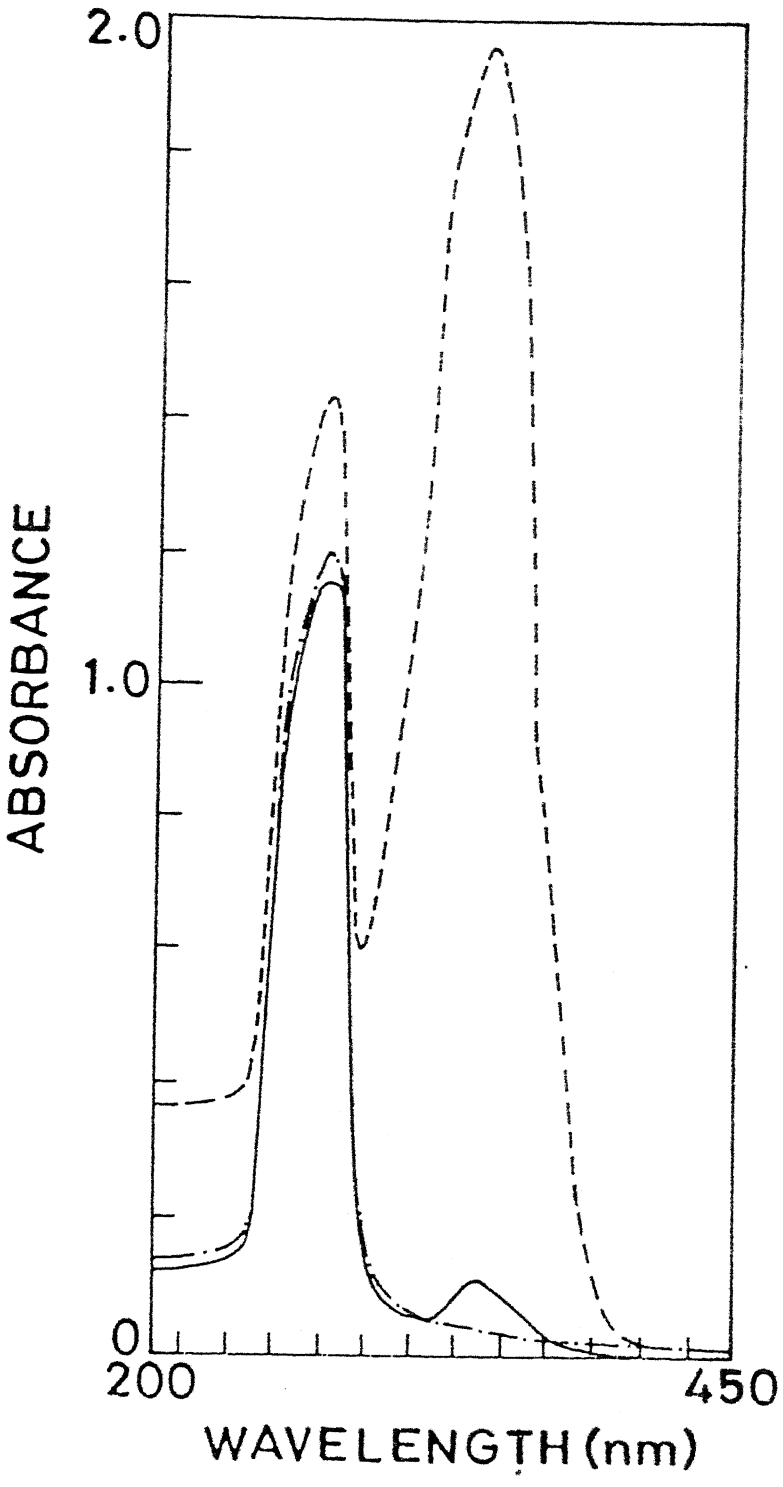
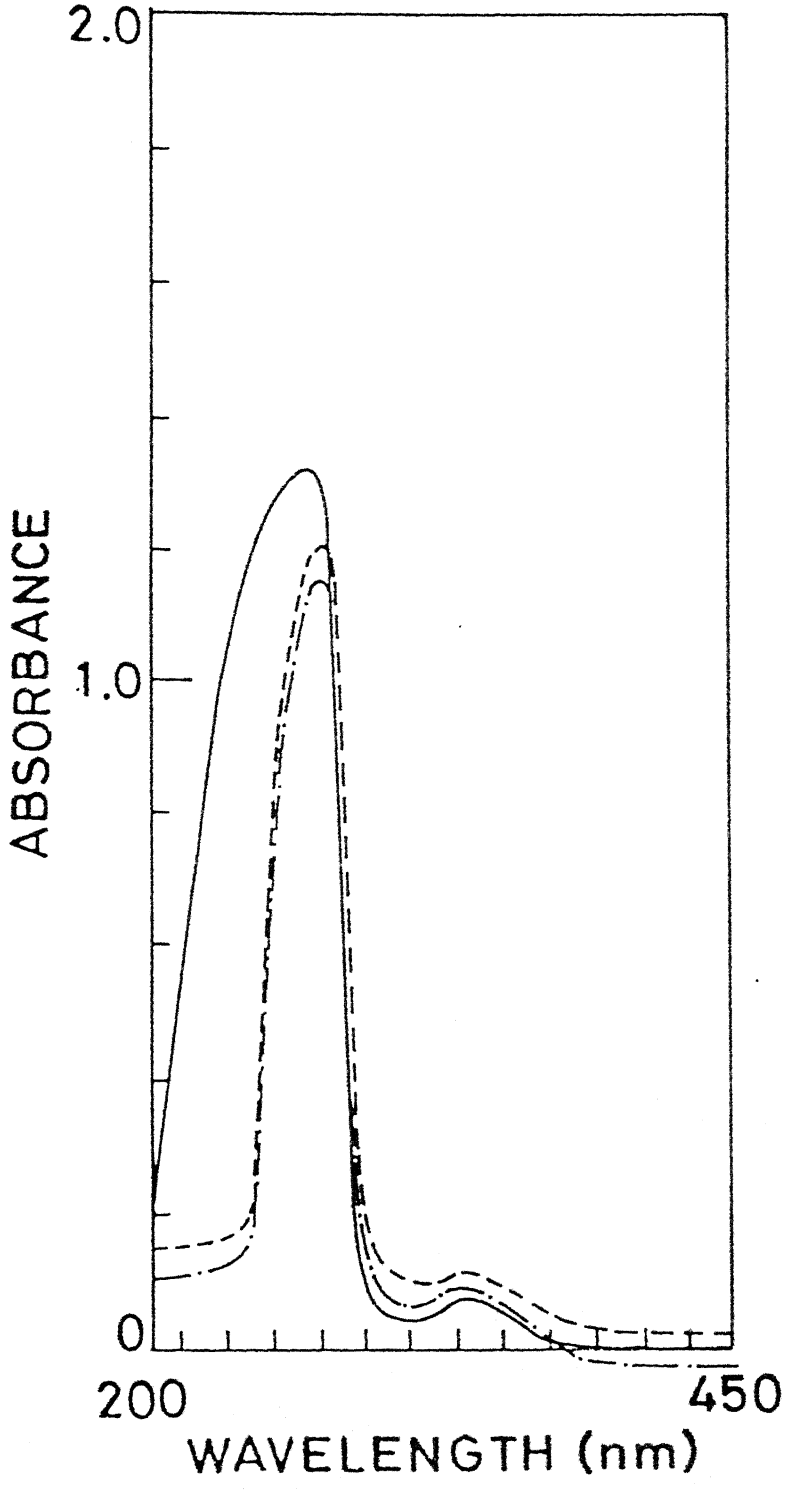


Fig.IV.6 Electronic absorption spectra of NAD^+, NADH and the product formed (after the completion of ADH(Y) catalysed reaction) in reverse micellar solution of CTAB in isooctane-chloroform (1:1, v/v) at w_o = 15.0 and pH = 10.0 (100 mM glycine-KOH).
———— product; - - - - - - - - - - - - - NADH;
-·-·-·-·-·-·- NAD^+.



ig.IV.7 Electronic absorption spectra of the product formed after completion of ADH(Y) catalysed reaction in aqueous buffer (pH = 8.3) and in reverse micellar solution at w_o = 15.0; pH = 10.0 and w_o = 20.6; pH = 10.0. Buffer used was 100 mM glycine-KOH.

———— product in aqueous buffer, pH = 8.3;

– – – – – product in reverse micelle, pH= 10.0, w_o = 15.0

— · — · — · product in reverse micelle, pH= 10.0, w_o = 20.6

Fig. III.8. In both aqueous and reverse micelle NAD^+ and NADH show absorption maxima at 260 nm and 260 nm and 340 nm respectively. The identical nature of spectra in aqueous and reverse micelle establishes that both NAD^+ and NADH are stable in the water pool inside reverse micelle of CTAB in isooctane-$CHCl_3$ (1:1, v/v).

To check the effect of water pool size on the product due to probable change in characteristics of water at different water pools, the spectra were recorded (Fig. IV.7) at two different water pools after the completion of the reaction. These spectra are identical to the spectra obtained in aqueous medium establishing thereby the identical nature of reaction in both media.

IV.3.6 Time Dependent Stability of ADH(Y) in Reverse Micelle

The residual activity of ADH(Y) has been measured at different time intervals by incubating the enzyme with or without coenzyme or substrate. It was observed that the enzyme looses around 98% of its activity with in one minute when it is incubated alone or with C_2H_5OH in the reverse micelle. On the other hand it lost only 13% of activity in one minute when it was stored in presence of NAD^+. It indicates that NAD^+ helps in maintaining an active conformation of the enzyme in the reverse micelle. This kind of observation where coenzyme provides the protection in reverse micelles has been observed in other cases.[36,37] These data indicate that coenzyme improve the stability of enzyme in both aqueous and reverse micellar media.

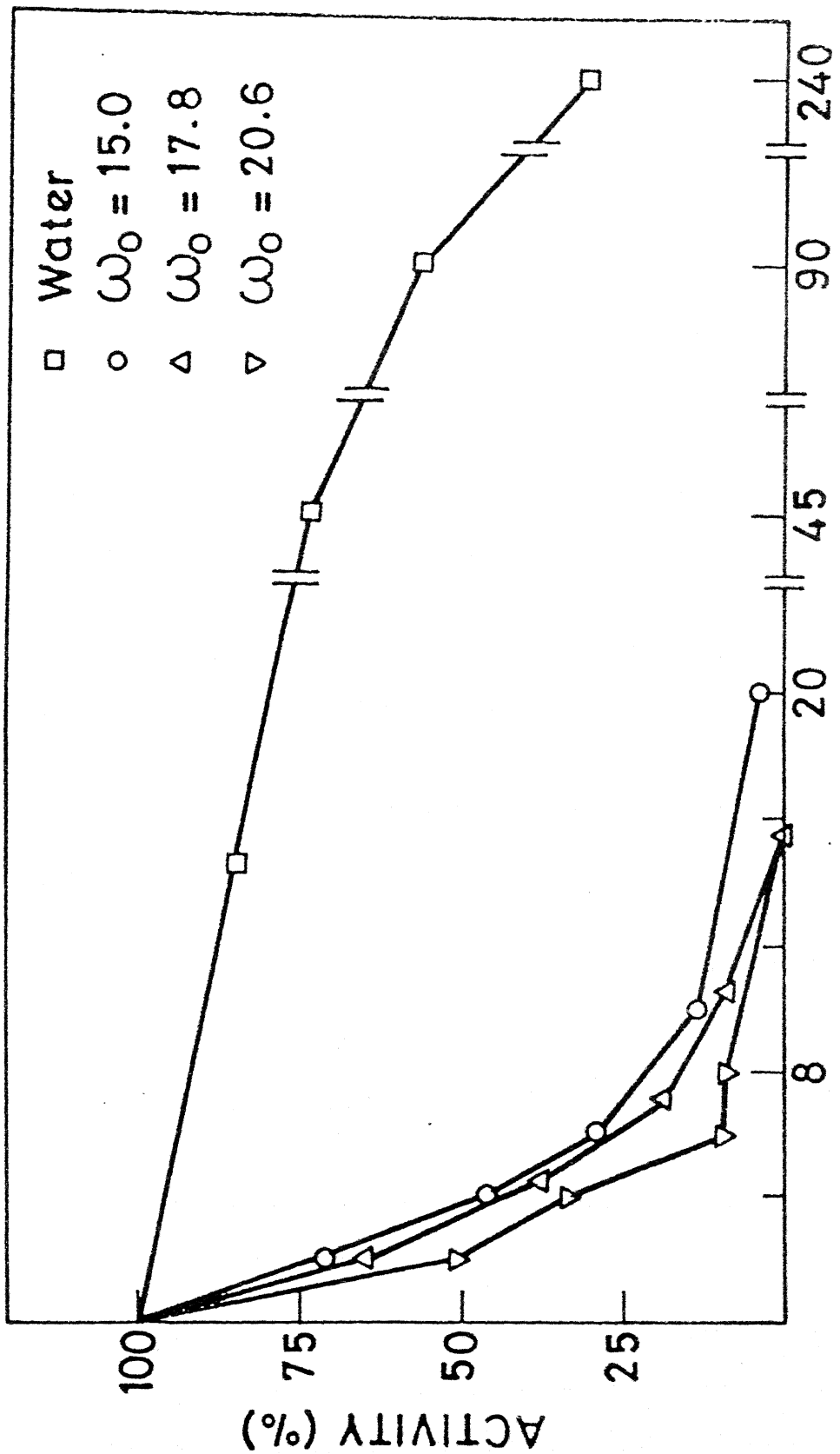


Fig.IV.8 Stability of ADH(Y) as a function of time in aqueous and 100 mM CTAB in isooctane-chloroform (1:1, v/v) reverse micellar solution in presence of coenzyme NAD^+ at different w_o. [A] = water, pH = 8.3 (pyrophosphate buffer); [B] w_o = 15.0, pH = 10.0; [C] w_o = 17.8, pH = 10.0; [D] w_o = 20.6, pH 10.0. The concentrations were $[NAD^+]^o$ = 0.5 mM; $[C_2H_5OH]$ = 0.1 M. Buffer used was 100 mM glycine-KOH.

Time dependent stability study of ADH(Y) with NAD^+ at different water pools has been investigated. Fig. IV.8 shows the plot of residual activity versus time of incubation. It may be noted that the stability of enzyme decreases with increase of water pool size. The enzyme at w_o = 20.6 looses 50% of activity in two minutes whereas at w_o = 15.0 the enzymes looses only 27% activity during the same period. These data show that ADH(Y) is unstable in CTAB reverse micelles in comparison to its stability in water. In aqueous buffer the enzyme retains 30% activity even after 4 hours whereas in reverse micelle it looses almost all of its activity by 20 minutes. At any time the activity of enzyme at higher water pools in lesser than the activity at low water pools. The enzyme shows most stability at the water pool at which it is most active. It indicates that the conformation at which the enzyme is most active, is also most suitable for better stability.

IV.3.7 Kinetic Characteristics of ADH(Y) in Reverse Micelles

Michaelis-Menten constants of ADH(Y) have been determined in reverse micellar solution of CTAB at pH = 10.0 and w_o = 15.0.

IV.3.7.1 Influence of Enzyme Concentration on Initial Velocity in Reverse Micelle

Effect of enzyme concentration on enzyme reaction velocity gives significant information about linear (or non-linear) relationship between velocity and enzyme concentration. Frequently non-linearity is due to an artifact in the assay

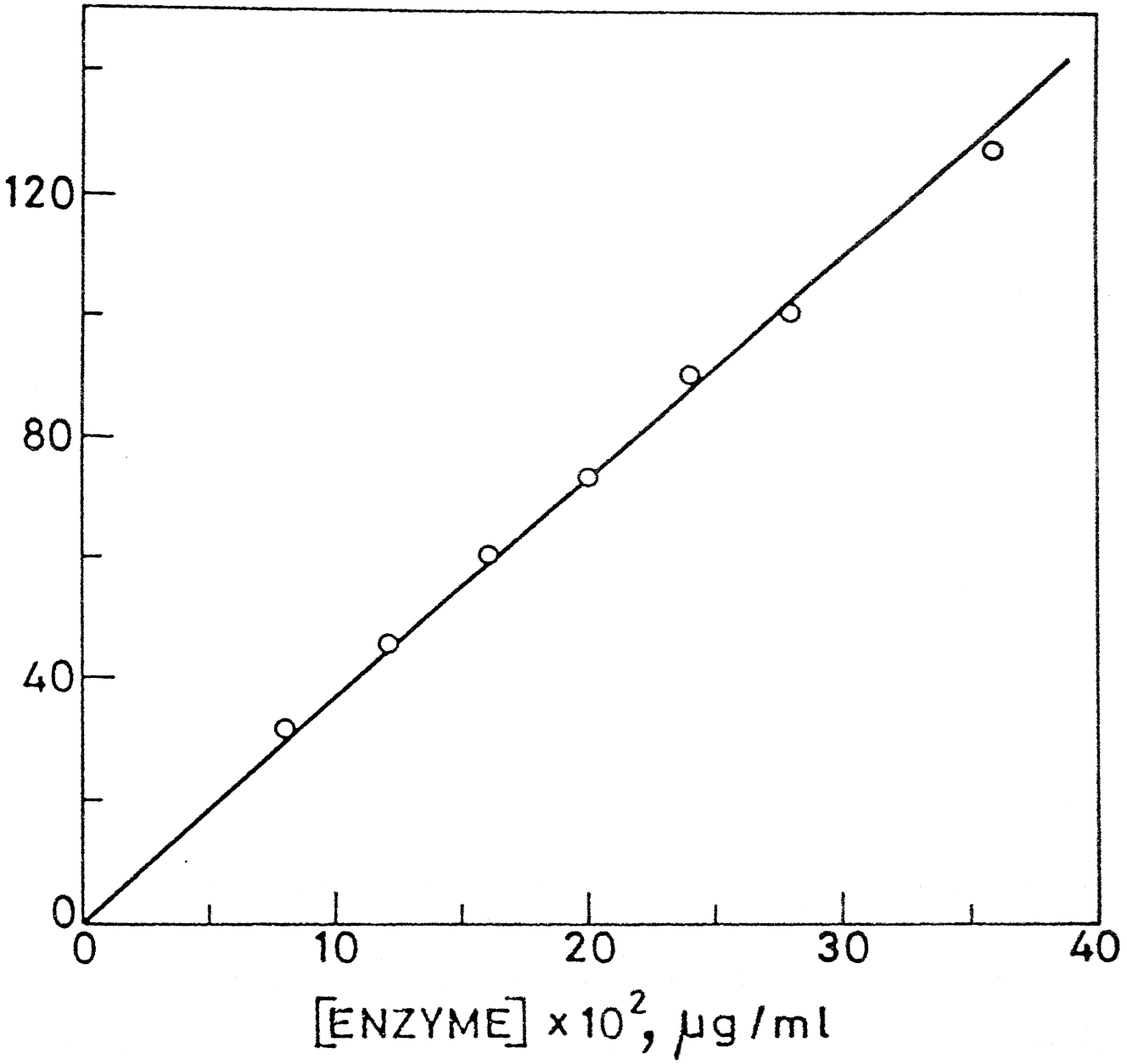
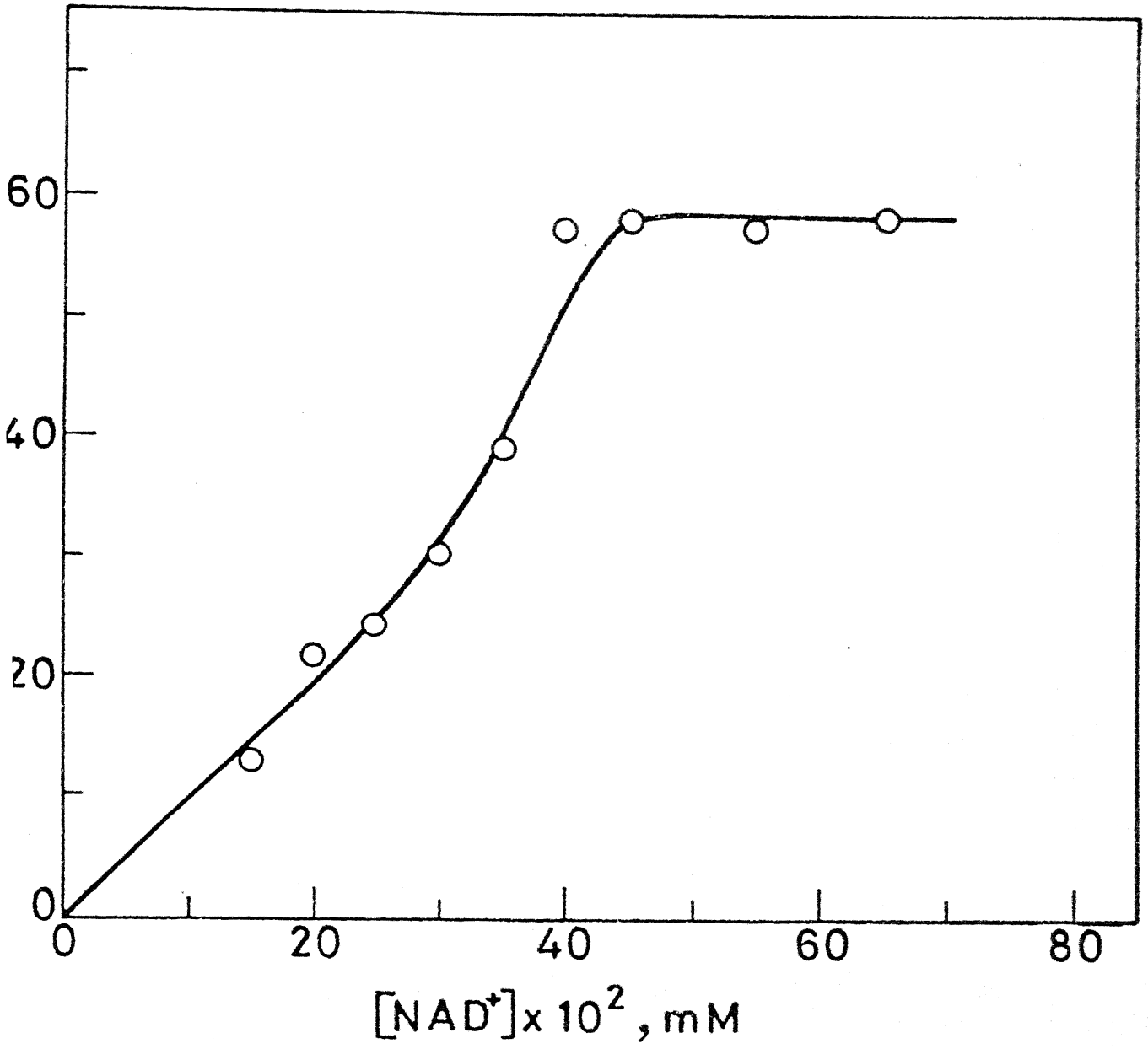


Fig.IV.9 Variation of initial velocity of enzyme reaction with ADH(Y) concentration in reverse micellar solution of CTAB in isooctane-chloroform (1:1, v/v) at pH = 10.0, w_o = 15.0, $[C_2H_5OH]$ = 100 mM, $[NAD^+]$ = 0.5 mM. Buffer used was 100 mM glycine-KOH.



g.IV.10 Effect of NAD^+ concentrations on ADH(Y) activity in reverse micellar system of CTAB in isooctane-chloroform (1:1, v/v) at w_o = 15.0, pH = 10.0. The concentrations were [CTAB] = 100 mM, $[^{\circ}C_2H_5OH]$ = 100 mM. The buffer was 100 mM glycine-KOH.

system, but there are examples where such behavior is characteristic of the enzyme itself. Fig. IV.9 shows the plot of initial velocity versus enzyme concentration in reverse micellar solution of CTAB in isooctane-$CHCl_3$ (1:1, v/v). The plot is a straight line and it passes through origin, indicating thereby that the molecules of enzyme act independently in reverse micellar solution.

IV.3.7.2 Effect of NAD^+ Concentration on ADH(Y) Activity in Reverse Micelle

The specific activity of the enzyme as a function of coenzyme concentration is shown in Fig. IV.10. Reaction rate initially increases linearly and then reaches a maximum value after which it remains unchanged. It shows that the effect of NAD^+ concentration on the activity of ADH(Y) in CTAB/issoctane-$CHCl_3$ (1:1, v/v) reverse micelle follows Michaelis-Menten kinetics. The velocity reaches saturation when NAD^+ concentration is equal to or more than 0.4 mM. The plot is linear upto 0.35 mM concentration of NAD^+.

IV.3.7.3 Effect of Substrate Concentration on ADH(Y) Activity in Reverse Micelle

The specific activity and substrate concentration profile keeping coenzyme concentration fixed at 0.4 mM is shown in Fig. IV.11. The study at low concentrations of C_2H_5OH could not be carried out due to appearance of turbidity in the solution. The concentrations range at which we could study the effect of

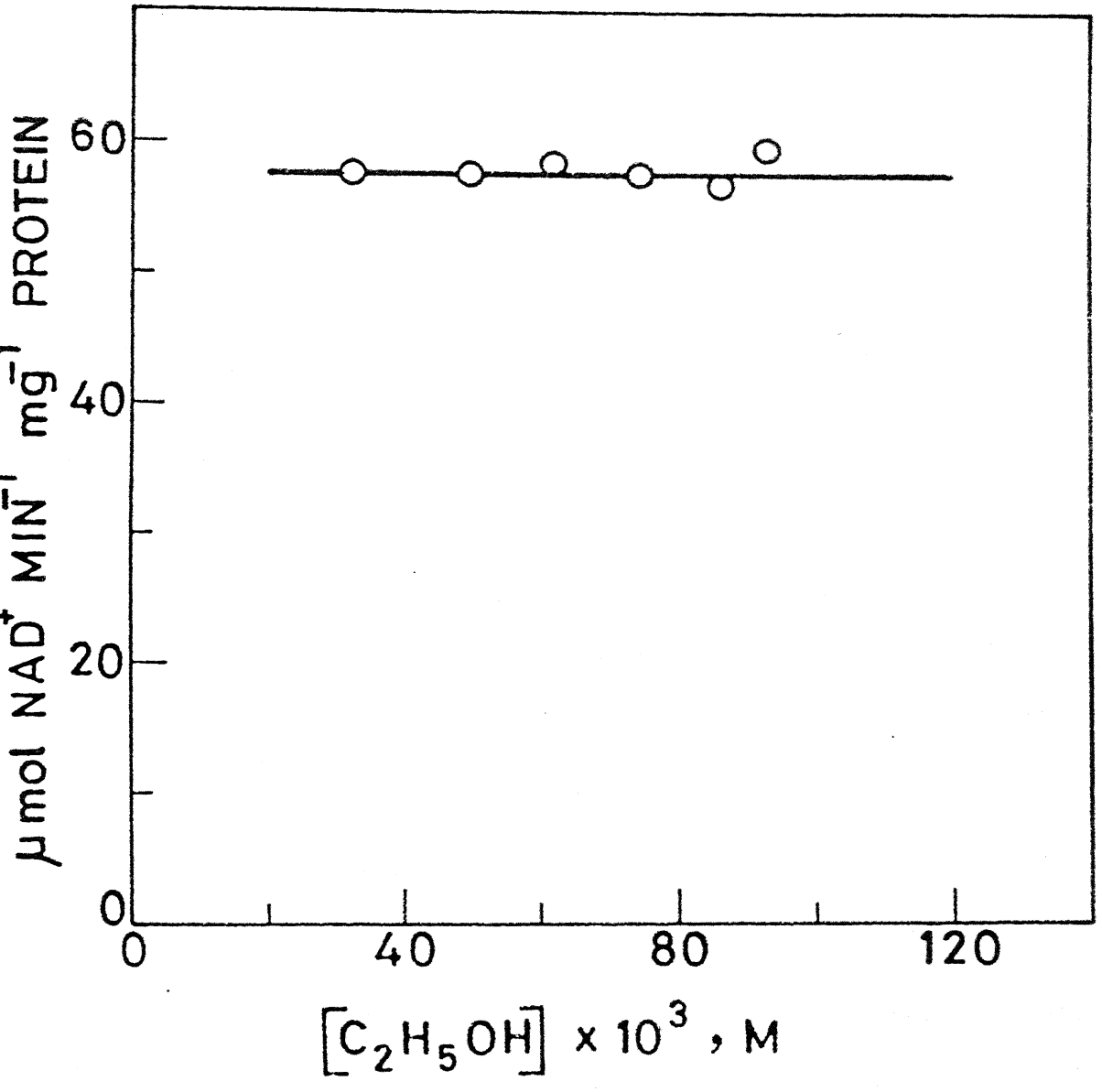


Fig.IV.11 Variation of ADH(Y) activity with C_2H_5OH concentrations in CTAB micellar system in isooctane-chloroform (1:1, v/v) at w_o = 15.0, pH 10.0. The concentrations were: [CTAB] = 100 mM; [NAD^+] = 0.4 mM. The buffer was 100 mM glycine-KOH.

C_2H_5OH concentration is the saturation concentration of the substrate.

IV.3.7.4 Lineweaver-Burk Plot

The plot of reciprocal of ADH(Y) catalysed reaction rate with reciprocal of NAD^+ concentration has been illustrated in Fig. IV.12. Kinetic parameters of ADH(Y) at w_o value 15.0 and pH 10.0 calculated from this data are summarized in Table IV.1. These parameters are reported in overall volume as well as in the water pool of reverse micelles.

The values of $(K_m)_{ov}$ and $(K_m)_{wp}$ are much higher than K_m in water. $(K_m)_{ov}$ value is found to be closer to K_m in water in comparison to $(K_m)_{wp}$. Since K_m is a good measure of the dissociation constant of the enzyme-substrate complex,[39-41] high value of K_m in reverse micelle indicates that the stability of ES complex in micellar medium is considerably less as compared to that in aqueous solution.

The possible reasons for the shift of pH optimum on alkaline side by ~ 2 pH units in reverse micelles than that in aqueous solution may be the following: (1) pK_a of certain amino acid residues at the active site of the enzyme changes due to conformational change on solubilization. (2) Characteristic of water in water pool are different from those in the bulk water.[43] (3) Charge on the micelle interier may influence the surface charge on enzyme molecule.

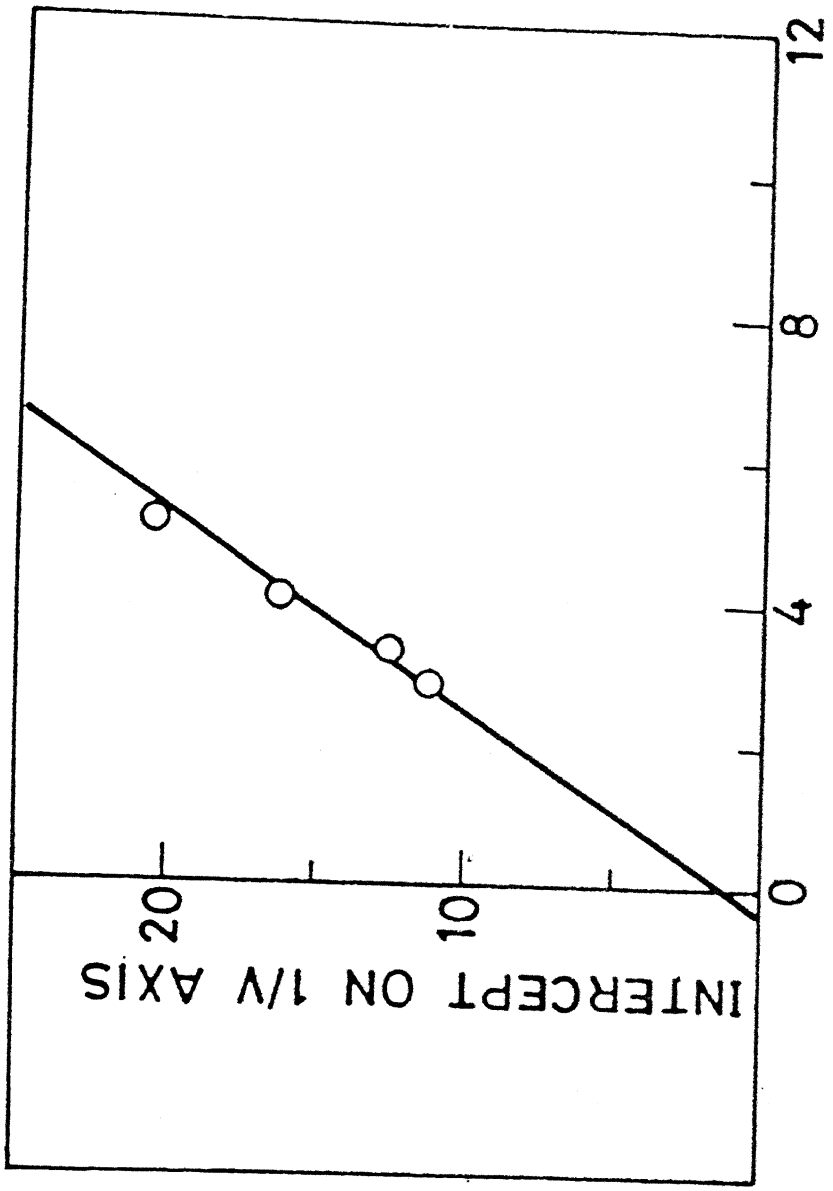


Fig.IV.12 Plot of inverse of initial rate with reciprocal of NAD^+ concentrations in 100 mM CTAB in isooctane-chloroform (1:1, v/v). w_o = 15.0 and pH = 10.0. Buffer was 100 mM glycine-KOH.

TABLE IV.1 Michaelis-Mention Constant of Yeast Alcohol Dehydrogenase

System	CTAB reverse micelle w_o=15.0, pH = 10.0	Standard deviation	Water pH = 7.15
K_m^{NAD}(ov) , mM	2.85	0.282	7.4×10^{-2}
K_m^{NAD}(wp) , mM	105.56	-	-
V_{max}, $\mu M\ min^{-1}$ (mg enzyme)$^{-1}$	57.5	-	115.93

ov = overall

wp = water pool

An optimum concentration of surfactant is required for the maximum activity of coenzyme in reverse micelles. It may be that at low surfactant concentrations the reverse micelles are not rigid enough to protect the enzyme from exposure to undesirable effects of organic solvent. On the other hand at high concentration of surfactant, increase in microviscosity of the water pool will decrease the reaction rate because of the restriction in the movement of enzyme molecules in the water pool.

One possible explanation for high value of K_m is that the ADH(Y) and NAD^+ species are located in the microemulsion in different environments. Such a separation would lead to reduced binding affinity[44] and hence an increased value of K_m. At pH 10 (the pH at which Michaelis-Menten constant has been determined) the enzyme molecule is expected to be negatively charge (isoelectric point of ADH(Y) in buffer is 5.4) and so it is drawn near to surface of reverse micelles and NAD^+ is repelled from surface by electrostatic interaction.

It appears that the charge on a micellar 'matrix' is an important factor that influences the efficiency of enzymatic catalysis is reverse micelles.

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CONCLUSION

1. Glucose-6-phosphate dehydrogenase from yeast though a relatively large enzyme (M.W. 212,000 dalton and 4 sub-units) has been successfully solubilized in mixed reverse micellar solution of AOT and Triton X-45 in n-heptane.

2. The enzyme reatins its activity in the hostile medium of organic solvent at specific conditions of degree of hydration (w_o), pH and surfactant concentration.

3. Striking feature of the present study is appearance of super activity in G-6-PDH in this microheterogeneous medium under optimum conditions. Super activity means higher enzyme activity compared to the activity in the aqueous medium

4. Yeast alcohol dehydrogenase which is a big enzyme of M.W. ~ 151,000 dalton and comprising 4 sub-units was solubilized in microheterogeneous systems of anionic surfactant and cationic surfactant in organic solvents. The optimum conditions for ADH(Y) where the enzyme shows maximum activity in reverse micelles were totally different than the optimum conditions in aqueous medium.

5. Another important finding is that ADH(Y) is superactive in reverse micellar solution of AOT in isooctane.

6. It appears that in general even for big and complex enzyme the activity in reverse micelles is highly regulated by different parameters like w_o, pH, surfactant concentration, substrate concentration etc.

7. Our investigations show that these complex enzymes maintain their conformational integrity and subunit-subunit interaction in reverse micellar media.

8. The study of time dependent stability on these enzymes show better stability with coenzymes as in aqueous medium.

9. The enzyme G-6-PDH in AOT-Triton X-45 in n-heptane and ADH(Y) in AOT/isooctane obey Michaelis-Menten kinetics upto specific concentration range of substrates or coenzymes.

10. The Michaelis constant (K_m) in reverse micellar media were calculated from Lineweaver-Burk plots which are higher than the value of K_m in aqueous medium. It reflects that enzyme-substrate complex in reverse micelles remains less stable compared to aqueous medium.

Though the surfactant and organic solvents are known to denature the enzymes individually, the enzymes seem to maintain their maximum activity, conformational integrity, stability and kinetic characteristics etc.in the microcaptive environment of surfactant aggregation in organic solvents. Since reverse micelles have some features similar to those of biomembranes, therefore display of super activity by G-6-PDH in AOT-Triton X-45 n-heptane and ADH(Y) in AOT/isooctane shows that enzyme <u>in vivo</u> may possess higher activity than actually found by <u>in vitro</u> studies in aqueous solution.

Micellar enzymology has tremendous potential in biotechnological applications of enzymes. Enzymes in reverse micelles find applications in fine organic synthesis, synthesis

of water insoluble compounds (steroids, lipids, fats etc), peptide synthesis, separation and isolation of proteins, energy conversion processes, bioluminescence assays, drug carrier in organisms, cryoenzymology, separation techniques etc. The enzymes G-6-PDH and ADH(Y) which are superactive in micro-heterogeneous system may find wide-spread applications in the years to come. The study of enzymes in reverse micelles mark a radical departure from traditional enzymology and this development may broaden the scope of biocatalysis in other areas of science.

LIST OF PUBLICATIONS

1. Super activity and stability of yeast alcohol dehydrogenase in non-aqueous solvents in presence of surfactants,
   S.S. Katiyar, Tapas K. De and Anil Kumar, Biochemical Journal (submitted).

2. Stabilization and super activity of yeast alcohol dehydrogenase entrapped inside reverse micelles in apolar solvent,
   Tapas K. De, Anil Kumar and S.S. Katiyar, Eur. J. Bio-Chemistry (submitted).

3. The phenomenon of super activity in yeast glucose-6-phosphate dehydrogenase in reverse micelles in apolar solvent,
   S.S. Katiyar and Tapas K. De, (manuscript under preparation).

4. Stabilization and kinetic behaviour of yeast glucose-6-phosphate dehydrogenase in microheterogeneous medium of surfactants in non-aqueous solvent,
   Tapas K. De and S.S. Katiyar, (manuscript under preparation).

5. Activity and kinetic study of yeast alcohol dehydrogenase in water microemulsion medium of cationic surfactant in non-aqueous system,
   Tapas K. De, Anil Kumar and S.S. Katiyar. (manuscript under preparation).

6. Study on the kinetics of triphenylmethane dyes and alkoxides in water entrapped into reverse micelles of nonionic surfactant in organic solvent,
   Tapas K. De, A. Kumar and S.S. Katiyar, (manuscript under preparation).

7. Catalytic effects on the reactions of carbocations with nucleophiles in microcaptive environment provided by reverse micelles of cationic surfactant,
   Tapas K. De and S.S. Katiyar, (manuscript under preparation).

PRESENTATIONS

1. Reverse micellar effect on the kinetics of carbocations and alkoxides,
   Sarvagya S. Katiyar and Tapas K.De, 3rd National Conference on Surfactants , Emulsions and Biocolloids , Aligarh ,India , 1987.

2. Stabilization and super activity of yeast alcohol dehydrogenase in microheterogeneous medium of reverse micelles.
   S.S. Katiyar, Tapas K. De and Anil K. Awasthi, Proceedings Seventh International Symposium on Surfactants in Solution, Ottawa, Canada (2-7 October, 1988).

3. Stability and super activity of yeast glucose-6-phosphate dehydrogenase in apolar solvent,
   Sarvagya S. Katiyar and Tapas K. De, 32nd IUPAC Congress, Stockholm in August 1989 (submitted)

VITAE

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Page	Line(s)		should read
10	12	molucules	molecules
12	14	satturation	saturation
16	20	miccellar	micellar
17	1	tempreture	temperature
40	17	bicompatible	biocompatible
43	4	is	are
45	10	mdeium	medium
112	2	characteristies	characteristics
131	2	loose	lose
131	14, 18,24	looses	loses
134	12	looses	loses
148	4	exploting	exploiting
162	16	looses	loses
164	5, 6, 10	looses	loses
164	12	in	is
			Enzyme